MECHANISM OF CAP-SNATCHING IN HANTAVIRUSES

By

Erdong Cheng

Submitted to the graduate degree program in Microbiology, Molecular Genetics and Immunology and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Dissertation Committee:

_____

Edward B. Stephens, Ph.D., Chairperson

_____

Joe Lutkenhaus, Ph.D.

_____

Jianming Qiu, Ph.D.

_____

Thomas M. Yankee, Pharm.D., Ph.D.

_____

Wenxing Ding, Ph.D.

_____

Benyi Li, M.D., Ph.D.

Date Defended: September 24th, 2014

The Dissertation Committee for Erdong Cheng

certifies that this is the approved version of the following dissertation:

MECHANISM OF CAP-SNATCHING IN HANTAVIRUSES

_____

Edward B. Stephens, Ph.D., Chairperson

Date approved: September 25th, 2014

Abstract

Hantaviruses are enveloped RNA viruses belong to the Family Bunyaviridae, which cause hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS) in humans. The hantavirus genome consists of three negative-strand RNA segments, S, M and L, which encodes nucleocapsid protein (N), glycoprotein precursor (GPC), RNA-dependent RNA polymerase (RdRp), and in some genus, a non-structural protein (NSs).

Hantaviruses initiate transcription by cap-snatching mechanism. Host mRNAs are processed by viral RNP and used as primers for transcription initiation. N proteins protect those cap donors and enrich them in P-bodies. We showed that in P-body deficient cells, capped oligos were also protected but not effectively used, suggesting that caps concentrated within P-bodies are preferred for cap-snatching. We also characterized the determinants for a capped transcript to be an efficient cap donor using test transcripts. We showed hantavirus more efficiently snatches caps from mRNAs not engaged in translation and the hantavirus RNP preferentially cleaves the cap donor mRNA after the G residue located 14 nucleotides from 5' cap. Moreover, better sequence complementarity between the 3' terminus of vRNA and cap donor favors cap-snatching. These results indicate hantavirus snatches caps from non-translating mRNA, and the viral RNP may recognize and process host-capped oligos based on position and complementarity of the annealing between to vRNA and capped oligos.

We also explored requirement of other viral proteins for RdRp function. We found N protein interacts with RdRp in virus-infected cells. We mapped the RdRp

binding domain to the N-terminal region of the N that excluded the RNA binding domain. Similarly, the N-binding pocket is located at the C-terminus of RdRp. We demonstrate that an N-RdRp interaction is required for RdRp function during the course of virus infection in the host cell.

Taken together, we characterized the preferred capped primer used for efficient cap-snatching. We also provided insights to the specificity of the hantavirus RdRp endonuclease, by showing N-RdRp interaction in the absence of RNA binding domain. Our findings have provided insight into the understanding of the mechanism hantavirus cap-snatching and will shed light on the identification of anti-viral targets for the treatment of hantavirus-caused diseases.

Acknowledgements

I would like to take this opportunity to thank Dr. Mohammad Mir and Dr. Joe Lutkenhaus who recruited me directly to the Ph.D. program of the Department of Microbiology, Molecular Genetics and Immunology at the University of Kansas Medical Center. I wouldn't be at this point in my science career without their kind help. I also thank Dr. Benyi Li, who gave me an opportunity to pursue my science career in the United States.

I will always be thankful to my advisor and mentor, Dr. Mohammad Mir, who gave me a chance to prove myself. I thank him for his guidance as well as support during my Ph.D. study. He not only inspired me but also enabled me.

I would like to express my sincere gratitude to Dr. Jiaming Qiu, for his encouragement and continuous, kind help over the past many years whenever I feel helpless during my research and study. I also show my deep appreciation to Dr. Edward Stephens, the chair of my defense committee, who spent an enormous amount of time correcting my dissertation and helping me preparing my defense.

Besides them, I would also thank the rest of my thesis committee: Drs. Thomas Yankee and Wenxing Ding for all the fruitful discussions as well as valuable suggestions during my thesis writing. I also thank my college Dr. Zekun Wang, who has always been joyful to work with.

Finally, I would like to dedicate this thesis to my parents, who gave me the chance to study in England at an early age; to my wife, who always has the faith in me and supports me; and to my yet-to-be born daughter, who is and always will be the motivation of my career.

Chapter I

Introduction

1. Hantaviruses and function of hantaviral proteins

Hantaviruses are negative-strand RNA viruses that belong to the family Bunyaviridae. Unlike other members in the family, which are carried by arthropods, hantaviruses are primarily rodent-borne. As of 2012, twenty-four hantavirus species have been discovered and based on their phylogenetic characteristics, have been divided into three clusters: Non-pathogenic viruses, hemorrhagic fever with renal syndrome (HFRS)-causing viruses, and hantavirus cardiopulmonary syndrome (HCPS)-causing viruses. The natural reservoir hosts of hantaviruses include rodents, shrews, moles and bats, and the pathogenic viruses can cause human diseases by transmitting via aerosolized rodent excreta. [1-3] Although person-to-person transmission of Andes virus (ANDV) has been reported [4] and blood transmission of Puumala virus has been documented[5]. Thus, the epidemics caused by hantaviruses are closely linked to the population dynamics of the rodent carriers in the certain geographical area. For example, the old world hantaviruses, Hantaan virus and Puumala virus are distributed in Asia and Europe respectively, whereas the new world hantavirus, Sin Nombre virus and Andes viruses, are only found in Americas. Interestingly, the pathogenic hantaviruses do not cause visible disease in rodents with the exception of ANDV. [6]

1.1 Diseases caused by hantavirus infection

In humans, pathogenic hantaviruses cause two acute diseases: Hemorrhagic fever with renal syndrome (HFRS) and Hantavirus cardiopulmonary syndrome (HCPS). HFRS is primarily caused by Hantaan virus (HTNV), Puumala virus (PUUV), Dobrava virus (DOBV) and Seoul virus (SEOV). This disease has a mortality of around 10% with renal specific infection indicated by proteinuria, hematuria and acute kidney injury [1-3, 7]. The course of the HFRS is highly variable and depending on the hantavirus genotype. However, most patients recover from the infection. On the other hand, HCPS is a more severe disease that results in pulmonary oedema and cardiogenic shock [8]. Myalgia, cough and diarrhea are more commonly present in HCPS than in HFRS [8, 9]. In some of the HCPS-causing hantaviruses, hemorrhagic and renal syndrome also presents alongside [9]. The mortality of HCPS is up to 40% [3, 8, 9]. In either disease, cytopathic effect of the virus has not been observed [1, 10]. Pathogenesis of hantavirus related disease appears to be closely related to β3 integrin, which is one of the cell surface receptor for pathogenic hantaviruses [10]. A common pathological characteristic of both HFRS and HCPS, hyperpermeability of the capillaries is thought be a result of β3 integrin inactivation [11-13]. This viral induced inactivation causes deregulation of VEGF receptor 2 (VEGFR2) on the vascular endothelial cell surface [10], which leads to the up-regulation of vascular endothelial growth factor A (VEGFA) followed by down-regulation of VE-cadherin down-regulation [10, 14, 15]. VE-cadherin is essential for the formation of endothelial tight junction and the loss of VE-cadherin results in loosen of cell

junction thus increased vascular permeability. The hemorrhaging associated with the infection is caused by viral induced thrombocytopenia and is also related to the deregulation of β3 integrins [7, 11, 12]. Although the mechanism of viral induced thrombocytopenia is still unknown, it has been suggested that infected endothelial cells may attach to quiescent platelets through interaction between hantavirus glycoproteins and the integrin αIIβ3 on the platelet surface [16]. In addition to vascular endothelial cells, hantaviruses can also infect tubular epithelial cells, glomerular endothelial cells and podocytes of the glomeruli in the human kidneys, leading to the disruption of cell junctions through ZO-1. This disruption might be the cause of renal manifestations observed during HFRS [17].

1.2 Hantavirus particle and viral proteins

The hantavirus particles are enveloped, around 120nm-160nm in diameter. The genome of hantavirus consists of three negative RNA segments that encapsidated by nucleocapsid proteins and contained inside the envelope [18, 19]. (Fig. 1A and B) Each segment of the viral RNA (vRNA) consists of an open reading frame (ORF) flanked by untranslated regions (UTRs) at both 3' and 5' terminus.(Fig. 1C) The terminus of each UTR contains a highly conserved nucleotides that are complimentary to each other to form a panhandle structure which thought to be important for transcription and replication[20, 21]. The ORF from S, M and L RNA segments encodes for nucleocapsid (N) protein, glycoprotein precursor (GPC) and RNA-dependent RNA polymerase (RdRp),

respectively. GPC undergoes co-translational cleavage to form Gn and Gc. In some hantavirus species, a small non-structure protein, NSs, is also encoded from the S-segment through an alternative ORF. (Fig. 1C)

Nucleocapsid protein, N

The nucleocapsid protein (N) is the most abundant protein in the virus. It plays a central role in the life cycle of the hantavirus. [22] N protein contains a highly conserved RNA binding motif that enables N protein to encapsidate the viral RNAs to form vRNPs.[23] In addition, as more functions of N protein are being revealed, the N protein has been suggested to be a multifunctional protein that regulates both viral and host functions such as cell survival, immune suppression, viral translation and replication as well as viral assembly.
The, N protein plays a crucial role in modulating apoptosis in the infected cells; it has been demonstrated to inhibit apoptosis induced by Daxx and SUMO-1 pathway through direct interaction [24-27] with Daxx and SUMO-1, respectively. In addition, a recent study also revealed that N protein inhibits the enzymatic activity of both granzyme B and caspase-3, which makes hantavirus infected cells resistant to cytotoxic lymphocyte-mediated apoptosis [28]. Second, the N protein also antagonizes host antiviral signaling by sequestrating the NF-κB in the cytoplasm [29]. Moreover, it has also been revealed recently that N protein from ANDV inhibits RIG-I/MDA5 mediated IFN signaling by interfering with TBK1 activation [30].

Third, the N protein is also able to regulate cellular mRNA translation and degradation apparatus. N protein has been shown to have a cap-binding domain

that enables N to bind host mRNA on cap and functionally replace the cellular eIF4F in the translation initiation complex to load the 40S ribosome to the N protein bound mRNA by directly interacting with the ribosomal subunit S19 [31-33] . Moreover, N also specifically enhances translation of the viral transcripts over the host *in cis* by recognizing and binding to the triplet UAG repeats on the 5' UTR of the viral mRNA with a higher affinity [34]. The N protein is also suggested to be the major actor on switching between mRNA transcription and genome replication through its RNA chaperone activity [35, 36]. The N protein of SNV is found to co-localized with cellular processing bodies (P-bodies), where host RNAs are sequestered and degraded. The stability of mRNAs that are targeted for degradation has been shown to increase either in the presence of N protein or during hantavirus infection [37].

Finally, N protein might also be crucial for initiation of virus assembly. In support of the idea, N protein has been shown to interact with the cytoplasmic tail of glycoprotein Gn, and nucleocapsid protein is able to stabilize Gn from autophagic clearance inside infected cell at later stages of viral infection. [38-40] In summary, hantavirus N protein performs different functions at different stages of the virus life cycle to facilitate propagation of the virus.

Glycoproteins, Gn and Gc

When expressed in the infected cells, the glycoprotein precursor is co-traslationally cleaved at a conserved WAASA motif by the cellular protease, yielding Gn and Gc. Both Gn and Gc have transmemberane domains and they

form heterodimers that protrude from the viral envelope [41-44]. During viral infection, the function of glycoproteins has been proposed to be important for receptor signaling and internalization. In support, the C-terminal tail of the Gn protein contains one or more conserved YxxL motifs that are known to direct receptor signaling within immune and endothelial cells [45]. Moreover, bioinformatics studies suggested that ANDV Gc protein is a fusion protein that may direct the viral fusion activity [46].

Several studies now suggest that Gn is important for viral assembly [39, 40, 47]. First, Gn was found to be degraded by autophagy at early stages of viral life cycle [40]. Second, Gn tails form a CCHC-type zinc finger which binds RNA or to each other [47]. And third, Gn is stabilized when N is accumulated in the cells at later stage of the viral life cycle and the cytoplasmic tail of Gn specifically interacts with N protein [39].

RNA-dependent RNA-Polymerase, (L-protein)

RNA-dependent RNA polymerase (RdRp), the largest protein produced by hantavirus (~250-280kDa), is the key polymerase for transcription and replication of the hantavirus genome. In order to perform both of the above functions, RdRp must have multiple enzymatic activities in addition to transcriptase and replicase. The additional activities may include a RNA endonuclease and possibly a RNA helicase activity. However, it is commonly known that RdRp lacks enzymatic activity necessary for capping and proof-reading. [48-50] It was long suggested from a study in bunyamwera virus that the L protein has the endonuclease

activity[51]. The structural and mutational studies using La Crosse (LACV, Bunyaviridae, genus *Orthobunyavirus*) RdRp revealed an endonuclease domain at its N-terminus that is similar to the endonuclease domain resides within PA subunit of the influenza polymerase [52]. In addition to the endonuclease domain, RdRp has a core structural polymerase domain that is conserved between both positive and negative strand RNA viruses. The overall structure of the core domain, which forms a right-handed fold, can be divided into three subdomains: thumb, the finger, and the palm [53, 54]. Sequence comparison revealed four highly conserved motifs (motif A-D) within the palm subdomain [54, 55]. The core residues essential for catalytic activity of polymerase resides within motif C, mutation of those residues results in loss of viral RNA synthesis in bunyamwera virus [56-58].

In addition, hantavirus RdRp is thought to be associated with the RNPs so that it is ready to initiate viral RNA synthesis immediately after the virus entry into host cell [59]. To date, there is no direct evidence for this model except that it was observed that hantavirus L protein, at least partially, is colocalized with the N protein in the Golgi region [60].

Non-structural proteins, NSS

In some hantaviruses, S segment also encodes a small non-structural protein from an overlapping reading frame, which possibly functions in interferon (IFN) antagonism [61].

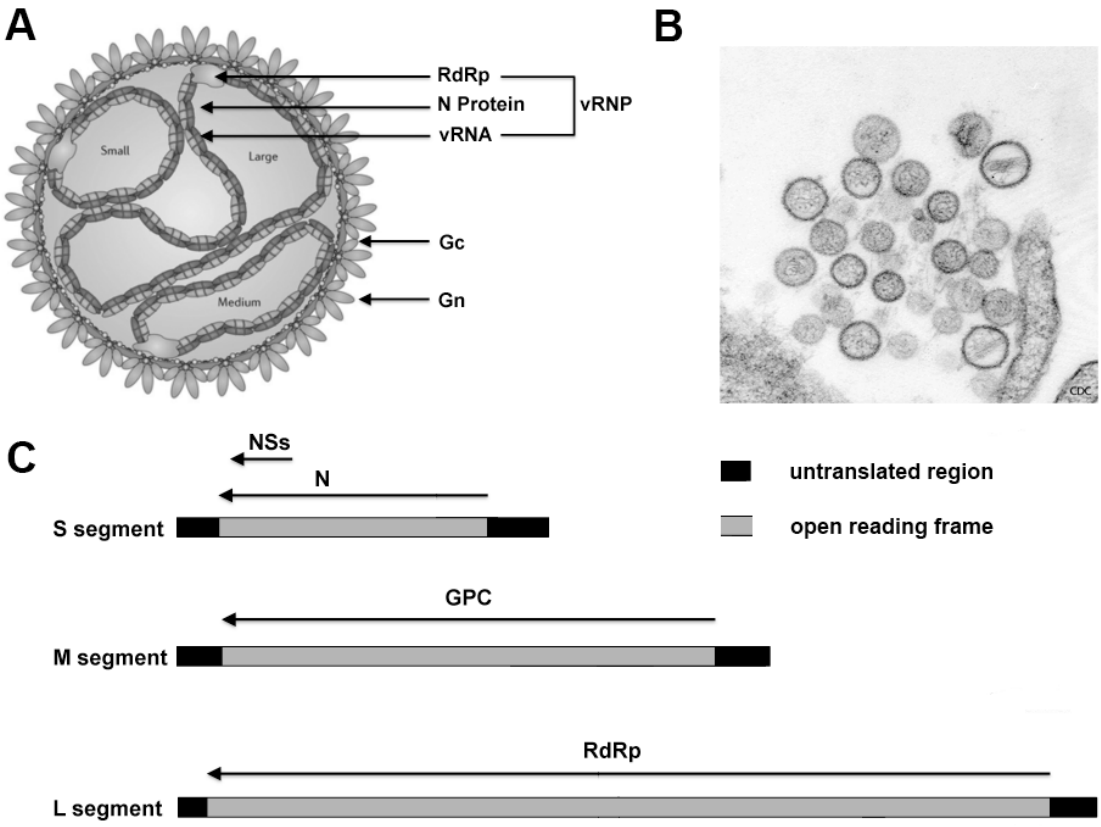

A
RdRp
N Protein
vRNA
vRNP
Small
Large
Medium
Gc
Gn
B
CDC
C
NSs
N
S segment
untranslated region
open reading frame
GPC
M segment
RdRp
L segment

Figure 1 Hantavirus particle and genomic organization.

(A) A schematic diagram of hantavirus particle showing RNP organization. (Modified from Vaheri *et al* 2013, ref 195) (B) Hantavirus particle under an electron microscope (image from CDC website) (C) Gene segments and protein expressed from each segments in hantaviruses.

2. Cap snatching, a unique transcription initiation mechanism of negative strand RNA viruses

The 5' cap structure (m7G) is an essential feature of eukaryotic mRNA required for mRNA stability and efficient translation. Viruses that infect eukaryotic cells rely heavily on the host cell machinery for translation. Thus, while some viruses, such as HCV, do not require capped mRNA to initiate translation, most viruses have to cap their mRNAs.

Some viruses cap their mRNA through capping enzyme, encoded either by host (DNA viruses) or virus itself (e.g. Dengue virus, vaccinia virus). However, some negative strand RNA viruses from Orthomyxoviridae, Bunyaviridae and Arenaviridae, which do not encode capping enzymes, have evolved a unique mechanism for transcription initiation called cap snatching. Cap-snatching was first described in influenza virus (Orthomyxoviridae) [62]. This process involves processing of host mRNA (in case of influenza, pre-mRNA) into short capped oligos and using them as primers for mRNA synthesis.

Cap-snatching mechanism has been extensively studied in influenza virus. *In vitro* and *in vivo* studies using either purified RNP or viruses revealed a preference on capped RNA primers based on sequence complementary to the 3’ end of vRNA template [63].

In addition, *in vivo* studies in infected Madin-Darby canine kindey (MDCK) cells also found internal priming at the 3’-penultimate residue, suggesting a

“prime-and-realign” mechanism for influenza virus cap-snatching. The heterotrimeric polymerase consists of PA, PB1 and PB2 subunit thought to be the key player for influenza cap-snatching. PB2 subunit binds the 5’ cap of host pre-mRNAs [64-66], which are subsequently cleaved after 10–13 nucleotides by the viral endonuclease, resides within N-terminus of PA subunit [67]. Further *in vitro* study using purified PA N-terminal (PA-Nter) fragment showed that the endonuclease domain within the PA subunit recognizes a 5’- CG-3’ motif from a 20-mer single strand RNA substrate for cleavage and changes on either the order or the composition of nucleotides in the 20-mer results in inefficient cleavage [68]. However, endonuclease activity of the PA-Nter is not affected by the presence of the cap at the 5’ end of the RNA substrate. However, when the same RNA substrate is incubated with the purified viral RNP, the cleavage efficiency is strongly increased in the presence of 5’ cap on the RNA substrate, whereas the recognition motif for cleavage is the same. Furthermore, analysis of the cleavage product showed that when guanine is present within 10-13 nt from the cap, RNP exhibits a dominating preference for it; whereas in the absence of guanine, RNAs are cleaved with high selectivity at the 12th nucleotide downstream from the cap. Thus, the combination of these studies suggest that although PA Nter endonuclease exhibits preference on G residue, the overall endonuclease specificity of RNP on a cap-donor is also determined by a combination of cap-binding-mediated distance measurement from the 5’ end of the RNA [68].

In the case of hantaviruses, evidence supporting prime and realign

mechanism has also been observed from sequencing data [69]. It is suggested that first C of the AUC repeats at the vRNA 3’ terminus pairs with 3’ G residue from the capped oligos derived from host mRNA; after successive addition of bases, the nascent RNA slips back a few bases and realigns with the complementary nucleotides at the 3' end of the vRNA [69]. Host mRNAs are processed for viral use at the replication site by the endonuclease activity of RdRp, thus producing capped primers of 10–15 bases for the transcription of viral mRNAs [52, 70].

In addition, the endonuclease activity similar to that of influenza virus PA subunit has also been demonstrated in LACV [52]. The amino acid motifs responsible for endonuclease activity are located at the N-terminus of the RdRp, and can be inhibited by an endonuclease inhibitor that specifically antagonizes the activity of PA.

However, unlike influenza virus, which replicates inside the nucleus and snatches caps from pre-mRNA, the hantaviruses entire life cycle is in the cytoplasm where only the cytoplasmic mRNAs are accessible for use as capped primers. It is not fully understood how cytoplasmic mRNAs are utilized by hantaviruses as primers, however, it has been suggested that acquisition of capped mRNA as primer by hantaviruses may involve the localization of viral proteins to cytoplasmic processing bodies (P-bodies), where they can access host mRNAs that are sequestered and possibly destined for degradation. The supporting evidence is that hantavirus N protein co-localizes with the P-body formed at the perinuclear region and during viral infection the stability of RNAs

targeted for degradation increase [37]. After N protein associates with the capped mRNA in the P-body, they are either been transported to the ER–Golgi intermediate compartment, a putative site for viral replication [71], or initiate viral RNA synthesis within the P-body right away [37].

3. P-body

Studies in the past have revealed that when the mRNA is no longer active in translation and targeted for sequestration or degradation, translationally repressed messenger ribonucleoproteins (mRNPs) form from the mRNA and associate with the cytoplasmic decapping machinery to form aggregated granules [72, 73]. These aggregates are named P-bodies (processing bodies or GW bodies).

3.1 P-body structure, formation and mRNA fate

Although complete protein composition of P bodies is unknown; P-body components identified to date can be roughly divided into three classes: First class includes proteins from mRNA decapping machinery, such as the decapping enzymes Dcp1, Dcp2, decapping activators Dhh1, Edc3, Lsm1-7, as well as the 5’ to 3’ exonuclease and the deadenylase CCR4/NOT [74-76]. These components form the core of the P-body and are conserved from yeast to mammals and are mostly members of the mRNA decay machinery. The second class of P-body components is more dynamic; the association of them with the core components depends on the organism of specific subset of mRNAs. For example, proteins involved in nonsense-mediated decay (NMD), which are not permanent components of the P-body, and are only found in P-bodies under certain conditions such as stress [77, 78].

The last component is the mRNA. [79, 80] It has been demonstrated that mRNA is required for the formation of the P-body, as RNase treatment *in vitro*

disrupted P-bodies purified from the cells [79].

Overexpression of a non-translating mRNA yeast also suggested P-body size and number are correlated to the amount of mRNA that is not participating in translation [79]. It is also suggested that mRNAs associated with P-bodies are not engaged in translation. Treatment of cells with cycloheximide that entraps ribosomes on the mRNA and depletes ribosome free mRNAs, results in the disappearance of visible P-bodies [73, 74, 79]. Conversely, the size of P-bodies increases when mRNAs dissociate from ribosomes [79, 81, 82]. Thus, one would speculate that translation initiation factors and ribosomes are absent in P-bodies and their associated mRNAs [75, 79, 83, 84]. However, one exception is eIF4E, the cap-binding subunit of the eIF4F initiation complex, although the capped mRNA in associated in P-bodies are translationally repressed [75, 84, 85].

The kinetic model of P-body formation is still unclear. Several lines of evidence suggest mRNPs must be translationally repressed and recruit cytoplasmic factors to form a P-body [72, 78, 79, 86-88]. It has been suggested that decapping accessory proteins Dhh1 and Pat1, serve as first activators of P-body formation followed by translation repression [86, 89-94]. Yeast strains lacking Dhh1 and Pat1 display defects in translational repression as well as P-body formation under stress while overexpression of Dhh1 and Pat1 promotes the formation of visible P-bodies [86]. However, although translational repression is clearly required for the assembly of an mRNP into a P-body, it may not be sufficient. In addition, Edc3 and Lsm4 were identified as factors required for P-body assembly from a yeast study, since deletion of both Edc3 and Lsm4 leads

to a complete failure of P-body formation without any effect on mRNA decay [87, 95]. In addition, single deletion of each factor did not affect P-body assembly, suggesting Edc3 and Lsm4 might involve in two separate complexes through interacting with different proteins, in this case, Dhh1 and Pat1, respectively, may independently promote P-body formation [87, 90, 93]. In mammalian cells, while Edc3 is highly conserved in the interaction domain [96], Lsm4 might be functionally replaced by Hedls or GW182 that contains predicted Q/N-rich motif similar to yeast Lsm4 [87, 95]. Knockout studies also confirmed the importance of the two proteins in promoting P-body assembly. [72, 97-99]

Once associated with the P-body, mRNAs may go into two fates: Firstly decapping/degradation and secondly return to translation. Three lines of evidence support decapping/degradation. First, many mRNA decapping factors are found concentrated in P-bodies. Second, mRNA decay intermediate is localized to P bodies followed by a stall or inhibition of decay [73]. Third, P-bodies change in size when mRNA decay pathway is perturbed [73-75, 86, 100]. Many observations demonstrated that specific mRNAs accumulated in P bodies under certain conditions re-associate with polysome when conditions alter [83, 101]. These evidences suggest instead of solely being a factory of mRNA decay, P-bodies serve as a point of mRNA turnover cycle where sequestered mRNAs are stored for either decay or translation depending on the environment. Thus P-bodies are highly dynamic structures with different flexible components that associate and dissociate at different rates, but in a regulated fashion, possibly depending on the fate of the specific mRNAs [75, 82].

3.2 P-body and Viruses

It is well known that viruses do not encode their own translation or even transcription machinery due to their coding capacity. Thus one important aspect of host-viral interaction is how virus interacts with host machinery for translation, transportation and degradation of mRNAs. Many studies have found components of P-bodies are important for the successful life cycle of some retro viruses and other RNA viruses, moreover, some viral proteins has been observed to accumulate within the P-bodies. This suggests that P-bodies may play important role in the virus life cycle.

With HIV, the P-body component DDX3 was found to be required for unspliced genomic RNA export from the nucleus, and targeting the unspliced mRNA to the P-bodies was thought to be important for packaging [102]. With the plant virus brome mosaic virus (BMV, (+) ssRNA virus), P-bodies have been suggested to be important for initiating genome replication, as many viral proteins, including RNA1-3, RdRp are found within the P-bodies [103]. In addition, P-bodies were also shown to associate with the membrane-bound BMV replication complex [104]. Similarly, P-bodies were observed to increase hepatitis C virus (HCV) by targeting the viral genome to the P-body by binding to a liver specific miRNA, miR-122 [80, 105].

Interestingly, viral proteins from negative-stranded RNA viruses also interact with the P-body. The influenza virus NS1 was found to interact with P-body through a core component RAP55 (Lsm14A). It was suggested that NS1

interacts and disrupts RAP55 associated P-bodies to release NP associated mRNAs that are targeted to P-bodies, thus restoring viral translation [106]. In hantaviruses (Bunyaviridae family) infected cells, N proteins are found to be associated with the P-bodies, and expression of the N proteins inside the cell increases the steady state level of the mRNA containing a pre-mature termination codon (PTC), a mutated stop codon in the middle of a ORF, which are targeted to degradation [37]. This observation suggested that N protein protects the 5' terminus of the capped mRNA sequestered within the P-body. N was speculated to remain bound to the caps during transcription and serve in translation initiation immediately following viral mRNA synthesis. Two lines of evidence supported the speculation: first, N is able to initiate translation by binding to the 5' cap and functionally replaces eIF4F [31]; this was in consistent with the observation that the host cap-binding protein within the translation initiation complex, eIF4E is also found to be associated with the P-body.[84] Second, bunyaviridae mRNA translation is coupled with transcription [107]. In conclusion, P-bodies play an important role in viral replication, and virus interacts with P-body to regulate host functions such as mRNA decay and translation to facilitate its own replication.

4. mRNA decay, decapping and viruses

In eukaryotes, mRNA decay mechanisms are evolved not only to serve as quality control to prevent production of malfunction or toxic proteins, but also as a way of protein regulation through altering mRNA half-life. The general mechanism of eukaryotic mRNA degradation can be divided into three types: deadenylation dependent 3' to 5' decay, endonucleolytic digestion, and specific quality control pathways [108]. RNA decay mechanisms, depending on the purpose, are regulated through distinct pathways. Current studies only revealed a small fraction of the network of pathways actually exist in the cell. The regulated RNA decay mechanism that has been described include nonsense-mediated mRNA decay (NMD), that mainly responsible for quality control of newly synthesized mRNAs; histone mRNA decay that exemplify a cell-cycle-regulated process and AU-rich element (ARE)-mediated mRNA decay, which is induced by destabilization elements in the 3' untranslated region (UTR) [108]. Due to the relevance of the dissertation, only NMD is discussed here.

4.1 Non-sense mediated mRNA decay (NMD)

Nonsense-mediated mRNA decay (NMD) exists in all eukaryotes to eliminate mRNAs that has pre-mature termination [109]. NMD is essential as a surveillance pathway to prevent translation of aberrant protein and accumulation of non-translation mRNAs [110]. This mechanism is translation coupled and prevents the potentially toxic effects of defective transcripts that are routinely generated during gene expression [111].

NMD is thought to target mainly newly synthesized mRNAs during the very first

round of translation. The newly synthesized mRNA normally has exon-junction complexes (EJCs) bound to their exon junction, and would be removed by ribosome during the first round of translation [111]. When mRNA contains a PTC before the exon junction, the EJC will remain bound to the mRNA when ribosomes fall off from the mRNA before removing the EJC, NMD is then activated [108]. Three conserved factors are critical for NMD: UPF1, UPF2 and UPF3 [112-114]. In mammals, PTC-induced NMD is initiated by interaction between cap-binding protein CBP80 and UPF1, the interaction complex in turn recruits SMG1 and contact with UPF2 and UPF3 within the EJC, triggering the phosphorylation of UPF1. The phosphorylated UPF1 then recruits SMG-5, SMG-6 and SMG-7, which mediates a series of mRNA degradation events including decapping, 5' to 3' decay and 3' to 5' decay [112, 115, 116].

In addition, recent studies revealed that NMD might also initiate independent of EJC or splicing [117, 118]. For example, immunoglobulin-ì mRNA is subjected to EJC-independent NMD even when PTC is located at the penultimate exon. The activation of this type of NMD depends on the length of 3' UTR.[117] Moreover, a study on Rous Sarcoma virus RNA found that unspliced viral RNAs, when inserted with a PTC, became substrates for the NMD pathway. This splicing independent NMD also requires UPF1 [119]. Furthermore, non-coding RNAs (ncRNA) can also be targets of NMD. The growth arrest-specific 5 (GAS5) ncRNA accumulates during growth arrest induced by serum starvation. A study showed that the ncRNA, GAS5, could be degraded specifically by NMD, as siRNA knockdown of UPF1 significantly increases the stability of GAS5 RNA

[120]. It has been proposed that P-bodies (described in earlier section) are the site where NMD is executed. In support of the concept, core NMD factors UPF1, UPF2 and UPF3 have been found associate with P-bodies [121]. It has also been proposed that UPF1 directs PTC-containing mRNAs to P-bodies and triggers decay [122].

4.2 Decapping and the impact on Bunyaviridae family viruses

When mRNA is targeted for degradation in either 5' -3' and 3'-5' fashion, decapping is always required. Three decapping enzymes has been shown to involved directly in the decapping process, Dcp2, Nudt16 and DcpS. Dcp2 and NUDT16 belong to the Nudix (nucleoside diphosphate linked moiety X) phosphatidyl hydrolase family, which catalyzes mRNA cap hydrolysis in the 5'-3' mRNA decay pathway in a metal-dependent reaction that releases m^7GDP and monophosphate-terminated mRNA.

Dcp2 is the first identified decapping enzyme, which is highly conserved in eukaryotes. The decapping activity resides in the central domain consisting of a Nudix fold structure. Dcp2 was reported to hydrolyze monomethylated, trimethylated and unmethylated capped RNA with different efficiencies *in vitro*. Dcp2 only cleaves capped RNA with longer than 25nt, it binds to the RNA and cleaves only cap structure. Dcp2 forms a complex with Dcp1 *in vivo*, and the *in vivo* activity of Dcp2 is critically dependent on Dcp1. Point mutations on Dcp1 without interfering Dcp1:Dcp2 association disrupt the decapping activity of Dcp1:Dcp2 complex *in vivo* [123, 124]. Nudt16 is another Nudtix motif containing

enzyme that has been demonstrated with decapping activity in vitro and in the cytoplasm of mammalian cells.[125]

In contrast, DcpS in contrast is a scavenger decapping enzyme that belongs to HIT protein family that catalyze residual cap-mRNA hydrolysis in an exosome mediated 3' to 5' decay pathway [126]. In both yeast and mammalian cells, decapping by Dcp1:Dcp2 complex has been demonstrated to be stimulated by interacting with several proteins, including Dhh1, Lsm proteins, Pat1, and enhancer of decapping (Edc) proteins. In yeast, Dhh1, Lsm 1-7 and Pat1 are found associated with the Dcp1:Dcp2 complex [91, 93, 94]. Depletion of any of these factors in *S. cerevisiae* results in accumulation of capped, deadenylated mRNAs, which suggests that they are critical for deadenylation-mediated decapping. Dhh1 is speculated to present the 5' end of the mRNA to decapping enzymes and Lsm 1–7 proteins were suggested to possibly recruit the decapping complex to deadenylated mRNAs [91, 92]. It is noteworthy that Dhh1 and Lsm1-7 are essential for initiating the formation of P-bodies. Decapping by Dcp1:Dcp2 complex plays a key role in NMD pathway, siRNA knockdown of Dcp2 results in impaired NMD *in vivo*. Although the mechanism of how NMD activates decapping is not understood, it has been shown that the core NMD protein, UPF1 is associated with the Dcp1:Dcp2 complex [127, 128].

5. Summary and Objectives

Hantaviruses have evolved a sophisticated strategy to initiate transcription in order to produce capped mRNAs. Although viral N protein has been shown to specifically enhance viral translation by recognizing the 5' triplet UAG repeats on the viral mRNA, efficient translation of viral transcripts in the absence of a cap structure has not been demonstrated. Thus the virus needs to acquire cap from the host mRNA (cap-donor) to not only achieve efficient translation, but also mRNA stability. It has been demonstrated that in influenza virus, non-viral lead sequence at the 5' of the viral mRNA mostly ends with GC or AGC, suggesting influenza virus prefers leader sequences with base complementarity to the most 3' nucleotide of the vRNA. The degree of complimentary to the 3' vRNA also determines the preference of specific cap-donor to be used as the primer [63]. In addition, cap-donor length is found to be 10 or 11 nt [63]. This is in consistent with the later in vitro study using purified endonuclease domain from PA (PA-Nter) to digest RNA substrates. PA-Nter recognize and digest RNA substrate at the 5'-CG-3' motif [68].

Similar observation has been found in bunyaviridae that the virus prefers to have the capped primer 10-20 nucleotides. Thus, it is of interest to determine whether hantavirus, a member of bunyaviridae, shows similar preference of cap-donor mRNA base on the complimentary between the nucleotides on the cap-donor and the vRNA.

Unlike influenza virus, hantavirus only replicate in the cytoplasm. Thus, the pool of the potential cap donors is only limited to the cytoplasmic mRNA. It is

important to find how hantavirus manages to snatch caps efficiently from the cytoplasmic mRNAs, since they are often engaged with the translation machinery. The observation that N protein co-localizes with the P-bodies and protects mRNA from decay leads to the speculation that hantavirus may acquire cap from a specific population of cytoplasmic mRNAs, which are sequestered and designated for degradation.

In addition, the study that characterizes the endonuclease activity of influenza polymerase subunit PA observed differences of endonuclease specificity between the purified PA endonuclease domain PA-Nter and the viral RNP. The study demonstrated the importance of the 5' cap to the endonuclease within the vRNP. Both potency and specificity increases when a 5' cap is added to the endonuclease substrate. The results suggested a difference in the RNA cleavage site choice might be guided by cap binding to the PB2 subunit [68]. In hantaviruses, the RdRp is expressed as a single protein. Although similar endonuclease activity has been observed from the N-terminal fragment of the RdRp, the activity suggested being non-specific. Moreover, the cap binding of RdRp has not been demonstrated, and the cap-binding motif has not been observed within RdRp [52]. Thus, further studies are required to demonstrate how hantavirus RdRp can also achieve specific cleavage of the mRNA substrate; and whether the specificity is induced by its cap binding, or cap binding ability or another cap protein, such as N protein.

Chapter II

Signatures of Host mRNA 5' Terminus for Efficient Hantavirus Cap Snatching

Abstract

Hantaviruses, similarly to other negative-strand segmented RNA viruses, initiate the synthesis of translation-competent capped mRNAs by a unique cap-snatching mechanism. Hantavirus nucleocapsid protein (N) binds to host mRNA caps and requires four nucleotides adjacent to the 5' cap for high-affinity binding. N protects the 5' caps of cellular transcripts from degradation by the cellular decapping machinery. The rescued 5' capped mRNA fragments are stored in cellular P bodies by N, which are later efficiently used as primers by the hantaviral RNA-dependent RNA polymerase (RdRp) for transcription initiation. We showed that N also protects the host mRNA caps in P-body-deficient cells. However, the rescued caps were not effectively used by the hantavirus RdRp during transcription initiation, suggesting that caps stored in cellular P bodies by N are preferred for cap snatching. We examined the characteristics of the 5' terminus of a capped test mRNA to delineate the minimum requirements for a capped transcript to serve as an efficient cap donor during hantavirus cap snatching. We showed that hantavirus RdRp preferentially snatches caps from the nonsense mRNAs compared to mRNAs engaged in translation. Hantavirus RdRp preferentially cleaves the cap donor mRNA at a G residue located 14 nucleotides downstream of the 5' cap. The sequence complementarity between the 3' terminus of viral genomic RNA and the nucleotides located in the vicinity of

the cleavage site of the cap donor mRNA favors cap snatching. Our results show that hantavirus RdRp snatches caps from viral mRNAs. However, the negligible cap-donating efficiency of wild-type mRNAs in comparison to nonsense mRNAs suggests that viral mRNAs will not be efficiently used for cap snatching during viral infection due to their continuous engagement in protein synthesis. Our results suggest that efficiency of an mRNA to donate caps for viral mRNA synthesis is primarily regulated at the translational level.

1. Introduction

Hantaviruses, members of the Bunyaviridae family, are transmitted to humans through aerosolized excreta of infected rodent hosts. Their infection causes hantavirus cardiopulmonary syndrome (HCP) and hemorrhagic fever with renal syndrome (HFRS)[129, 130], with mortalities of 50% and 15%, respectively. The spherical hantavirus particles harbor three negative-sense genomic RNA segments, S, M, and L, within a lipid bilayer[131]. The mRNAs derived from S, L, and M segments encode viral nucleocapsid protein (N), RNA-dependent RNA polymerase (RdRp), and glycoprotein precursor (GPC), respectively. The GPC precursor is cleaved into two glycoproteins, Gn and Gc. The characteristic feature of the hantaviral genome is the partially complementary sequence at the 5' and 3' termini of each of the three genome segments that undergo base pairing and form panhandle structures[132-134]. N is a multifunctional protein playing a vital role in multiple processes of the virus replication cycle and has been found to undergo trimerization both in vivo and in vitro [29, 31, 36, 135-143]. N specifically encapsidates the three viral genomic RNAs into nucleocapsids which are packaged into virions.

The sequence of L segment RNA has been determined for about 20 viruses in the Bunyaviridae family, including nine hantaviruses. Except for the tospovirus and nairovirus, the RdRps of all other bunyaviruses are of a similar molecular mass (~250 kDa). The requirement of both RdRp and N for replication/transcription of the viral genome has been demonstrated for both hantaviruses and other bunyaviruses [137, 144-146]. Using a green fluorescent

protein (GFP) fusion protein, the localization of Tula hantavirus RdRp has been found to be perinuclear. The punctate expression pattern of the L-enhanced GFP (EGFP) fusion protein has led to the suggestion that RdRp is membrane associated [60, 64]. The 5’ and 3’ termini of the hantaviral genome contain untranslated regions (UTRs) of various lengths. Assays in which reporter genes have been flanked by these UTRs have shown that promoters for viral RdRp are located in these critical UTR sequences. In the Orthobunyavirus Bunyamwera, base pairing of 5’ and 3’ termini of the viral genomic RNA was found to be required for the synthesis of RNA by viral RdRp [147]. Studies on the characterization of the influenza A virus promoter have suggested a corkscrew-like secondary structure formed by the base pairing of partially complementary 5’ and 3’ ends of the viral genome [148].

The RdRp from segmented negative-sense RNA viruses requires a capped RNA primer to initiate the transcription [149-153]. The capped RNA primer is generated from the 5’ terminus of host cell mRNA by the “cap-snatching” mechanism, which has been well characterized for the influenza virus [62, 66, 154-156]. Although the knowledge about the sequence, length, and structure of the 5’ mRNA terminus that donates the primer is rather limited, most common cap donor mRNAs are cleaved 15 nucleotides downstream of the cap, with a variation of 10 to 20 nucleotides [133, 150, 151, 154, 157-161]. The use of capped primers following a “prime and realign” mechanism has been suggested for the Bunyaviridae transcription initiation [69]. Transcription termination signals have been identified in Hantaan and Sin Nombre virus (SNV) mRNAs. SNV S

and L segment mRNAs are not polyadenylated; however, M segment-derived mRNA is polyadenylated and synthesis is terminated at a (U)8 polyadenylation transcription termination signal [162].

A cap-snatching mechanism similar to that in influenza virus has been proposed for all minus-strand segmented RNA viruses, including the bunyaviruses and arenaviruses, although their RdRps are structurally different and they replicate at different locations inside the host cell. Unlike the situation in influenza virus, the RdRp of bunyaviruses and arenaviruses is encoded by one rather than three genes. Recent studies have suggested that RdRp from bunyaviruses and arenaviruses harbors the endonuclease domain at the N terminus, and its endonuclease activity has been demonstrated [163]. Moreover, influenza viruses carry out cap snatching and transcription in the nucleus of infected cells, whereas bunyavirus and arenavirus transcription and genome replication are cytoplasmic. Unlike influenza virus, the viruses carrying out cap snatching in the cytoplasm have to compete with the cellular RNA degradation machinery, which actively removes caps and degrades cellular transcripts after the completion of translation.

The eukaryotic mRNA degradation machinery follows two general decay pathways, both of which begin with the shortening of the 3’ poly(A) tail by a process known as deadenylation [164]. Following deadenylation, mRNAs can be degraded by a 3’-to-5’ exosome under the control of peptides of the SKI complex. Alternatively, after deadenylation, mRNAs can be decapped by the Dcp1/Dcp2 decapping enzymes, followed by 5’-to-3’ degradation by exonuclease XRN1 [77,

165, 166]. Decapping and XRN1-dependent 5'-to-3' decay form the predominant pathway for the degradation of cellular mRNAs. Moreover, the components of this pathway, including decapping enzymes Dcp1/Dcp2, exonuclease XRN1, and other peptides that function in mRNA degradation and regulation, are located in discrete cytoplasmic foci termed processing bodies (P bodies) [77, 165, 166].

In addition to these two mRNA degradation pathways, eukaryotic cells also use elegant mRNA surveillance or quality control mechanisms to ensure the translation of error-free mRNAs. Among these, the most prevalent and well-characterized mechanism is the nonsense-mediated mRNA decay (NMD) pathway, which recognizes and degrades mRNAs containing premature translation termination codons (PTCs) [167]. Premature translation termination leads to the assembly of the surveillance complex on mRNA, which triggers NMD. The surveillance complex is composed of the UPF1 to three proteins and four additional NMD effectors (SMG1 and SMG5 to 7) [168-170]. Assembly of the surveillance complex recruits the decapping enzymes and XRN1, but it can also accelerate the deadenylation and 3'-to-5' degradation by the exosome and the SKI complex [171, 172]. The enzymes that function in general mRNA decay also function in NMD, and the mRNA molecules containing PTCs are targeted to P bodies for rapid decay [168-170].

We have recently found that SNV N protein resides in cellular P bodies and also binds specifically to the mRNA 5' caps [32, 36, 37]. This specific interaction prevented the 5' caps of cellular mRNAs from degradation by the cellular decapping machinery. The rescued 5'-capped oligoribonucleotides were stored in

P bodies by N and were later used as primers by the hantavirus RdRp [37]. We reported that 5’-capped mRNA oligonucleotides sequestered in P bodies by N were at least 180 nucleotides in length [37]. The mechanism generating the shorter primers of appropriate length and specificity is still unknown. In this paper, we examine the characteristics of the 5’ terminus of a capped test mRNA to delineate the minimum requirements for a capped transcript to serve as an efficient cap donor during cap-snatching mechanism of transcription initiation by the Sin Nombre virus RdRp.

2. Materials and Methods

Oligonucleotides, enzymes, and other reagents. PCR primers were from Integrated DNA technologies. All restriction enzymes were from New England BioLabs. Platinum PCR Supermix was from Invitrogen. Phusion high-fidelity DNA polymerase was from NEB. RNA purification reagents were from Qiagen, and reverse transcription reagents were from Invitrogen. Power SYBR green PCR master mix was from Applied Biosystems. TA cloning reagents were from Invitrogen. All other chemicals were purchased from Sigma. The reagents for 5′ rapid amplification of cDNA ends (RACE) were purchased from Roche Applied Science.

Constructs. The plasmid pCDNAGFP expressing green fluorescent protein (GFP) was PCR amplified from plasmid pEGFP-C1 (Clontech) using a forward primer, 5′-*GATTAT**GCTAGC**ATGGGGTCTCATGGCGAGGA*-3′, and a reverse primer, 5′-*GTATT**CTCGAG**TTATCTAGATCCGGTGGATCCC*-3′ (boldface and italics indicate restriction sites). The PCR product was gel purified, digested with NheI and XhoI restriction enzymes, and cloned between the same restriction sites in pCDNA3.1+ vector (Invitrogen). The plasmid pCDNAGFPns, which does not express GFP due to two substitution mutations, was cloned in pCDNA3.1+ using the forward primer 5’-*GATTAT**GCTAGC**ATGGGGT**GA**TCATGGCGAGGA*-3′ and the reverse primer described above. The plasmid pCDNAGFPnsG0, expressing a GFP mRNA harboring five substitution mutations at the 5′ untranslated region (UTR) of mRNA, was constructed by generating a PCR product from pCDNAGFPns plasmid using forward primer F1, 5’ –

AGTGTATCATATGCCAAGTAC-3', and reverse primer R1, 5′-GGATAAGGGAGTAAGGAGTGGGTTGTGTA-3'. Similarly, another PCR product was generated from pCDNAGFPns plasmid using forward primer F2, 5′-TACACAACCCACTCCTTACTCCCTTATCC-3′, and reverse primer R2, 5′-TCTAGACTCGAGTTACTTGTACAGCT-3′. The two PCR products were gel purified and mixed together. The mixture was used as the template and a third PCR product was generated using forward primer F1 and reverse primer R2. This final PCR product was again gel purified, digested with NdeI and XhoI, and cloned between the same restriction sites in pCDNA3.1+ backbone. Using this cloning strategy, the mutations were incorporated through forward primer F2 and reverse primer R1. The same strategy was used for the construction of other plasmids (see Fig. 9A and Fig. 10A). In all these constructs the same forward F1 and reverse R2 primers were used. However, the sequences of reverse R1 and forward F2 primers were different, depending upon the type of mutation (Table 1).

Reverse transcription and real-time PCR. Vero E6 cells in six-well plates were transfected with the plasmid of interest, using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Eight hours posttransfection, cells were infected with Sin Nombre virus (strain 77734, a gift from Brian Hjelle, University of New Mexico) at a multiplicity of infection (MOI) of 1.0. Cells were lysed 48 h postinfection, and total RNA was purified using the RNeasy kit (Qiagen), including treatment with RNase-free DNase I (Qiagen), following the manufacturer's protocol. Two micrograms of total RNA from each well was

reverse transcribed using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen) and a primer specific to the viral S-segment mRNA (5′-ACTAAAGCCAATCACACCCATGACA-3′) in a total volume of 20 µl. Two microliters of the resulting cDNA was used in 20-µl real-time PCRs. The relative quantification method was used for real-time PCR using an ABI Prism 7700 sequence detection system following the manufacturer's instructions (Applied Biosystems). Fold change in mRNA levels and standard deviation were calculated by the relative quantification method, which is described in detail in the ABI instruction manual (http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_040980.pdf). We used a universal primer set with a forward primer (5′-TGGCTAACTACACAACCC-3′) and a reverse primer (5′-ATGGTCATCAGGTTCAATCC-3’) to amplify 290 nucleotides from the 5′ terminus of viral S-segment mRNA. This universal primer set was used in all real-time PCRs reported in this paper, unless otherwise stated. The forward primer is complementary to the 5′ terminus of GFP mRNA, which is expressed from the transfected plasmid, and the reverse primer is complementary to the open reading frame of viral S-segment mRNA. This primer set will generate products only if the viral S-segment mRNA has snatched a cap from the GFP mRNA (discussed in more detail in Results). Amplification of β-actin mRNA as an “internal control” was carried out using a forward primer, 5′-CCATCATGAAGTGTGACGTGG-3’, and a reverse primer, 5′-GTCCGCCTAGAAGCATTTGCG-3’, as previously reported[37]. To ensure the

amplicon specificity of each primer set, the PCR products were subjected to melting curve analysis followed by sequential agarose gel electrophoresis. The efficiency for amplification of the target (5′ terminus of the viral S-segment mRNA) and the internal control gene (β-actin) was examined using serial dilutions of cDNA. The mean difference between threshold cycle number values was calculated for each cDNA dilution. The mean difference values corresponding to each dilution were plotted and fit to a straight line with a slope of <0.1. After this validation test, the levels of S-segment mRNA which have snatched caps from the test mRNA expressed from the transfected plasmid in Vero E6 cells were calculated following normalization to the β-actin mRNA levels and expressed as relative units. Intrinsic steady-state levels of GFP reporter mRNA in transfected cells were monitored by real-time PCR analysis using a forward primer, 5′-CACATGAAGCAGCACGACTT-3′, and a reverse primer, 5′-AGTTCACCTTGATGCCGTTC-3′. This primer set is specific to the GFP open reading frame.

TA cloning. During cap snatching, hantaviruses typically cleave the host cell mRNA at a G residue located 8 to 17 nucleotides downstream of the terminal cap [69]. To determine whether the caps derived from the mRNAs expressed from transfected plasmids (pCDNAGFP/pCDNAGFPns) exhibit these hallmarks of correct cap snatching, we sequenced the cap-viral UTR junctions of viral S-segment mRNAs, which have obtained their caps from either GFP mRNA or nonsense GFP mRNA. Vero E6 cells were transfected with either plasmid pCDNAGFP or plasmid pCDNAGFPns, followed by viral infection 4 h

posttransfection. Cells were lysed 48 h postinfection, and total RNA was purified and reverse transcribed using a primer specific to the S-segment mRNA, as described above. The cDNA was PCR amplified using a primer set shown in Fig. 2A. The PCR product was cloned using a TA cloning kit (Invitrogen) by following the manufacturer's instructions. Plasmid DNA was purified from 20 randomly selected clones and sequenced in the region corresponding to the cap-UTR junction, as previously reported[37].

siRNA knockdown. To substantiate the role of P bodies in hantavirus cap snatching, two essential P-body components, GW-182 and Ge-1, were downregulated by small interfering RNA (siRNA) transfection. The GW-182, Ge-1, and control siRNAs were purchased from IDT. The sequences for the GW-182 siRNAs were 5′-GGAAUGUUACAAGACAAACGAAUGG and 5′-CCAUUCGUUUGUCUUGUAACAUUCCUA-3′. The sequences of the Ge-1 siRNAs were 5′-GGAUGUUAGCCAGAUCAAGCAGGGC-3′ and 5′-GCCCUGCUUGAUCUGGCUAACAUCCAC-3′. Both GW-182 and Ge-1 siRNAs were transfected at a final concentration of 50 nM each into monolayers of Huh-7 cells (a gift from Yu-Jui-Yvonne Wan, KUMC) seeded in six-well plates using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Control siRNA was similarly transfected into control wells. Twelve hours after transfection, GW-182 and Ge-1 siRNA or control siRNA was retransfected together with 4 μg of pCDNAGFPns plasmid. The effect of siRNA knockdown on the expression levels of GW-182 and Ge-1 proteins was verified 24 h after first transfection by Western blot analysis, using either anti-GW182 or anti-Ge-1

antibodies (Santa Cruz). Cells were infected with SNV at an MOI of 1.0, 24 h after first transfection. Cells were lysed 36 h postinfection, and total RNA was purified and reverse transcribed using a primer specific to the S-segment mRNA, as mentioned in the “Reverse transcription and real-time PCR” section above. The effect of siRNA knockdown on hantavirus cap snatching was monitored by quantitative estimation of caps snatched from the GFPns mRNA by the SNV RdRp, using real-time PCR analysis, as discussed in the above “Reverse transcription and real-time PCR” section.

To determine the effect of a P body on the protection of mRNA caps by SNV N protein, monolayers of Huh-7 cells were either mock transfected or transfected with both GW-182 and Ge-1 siRNAs at a final concentration of 50 nM each. Twelve hours posttransfection, cells were retransfected with GW182 and Ge-1 siRNAs along with 4 μg each of plasmids pCDNAGFPns and pCDNA-SNVN. Cells were lysed 24 h after plasmid transfection, and total RNA was purified. Two micrograms of total RNA was reverse transcribed using random primers. The cDNA generated from both mock- and siRNA-transfected cells was used in real-time PCR analysis to quantitatively estimate the 5′ terminus of GFPns mRNA, using a forward primer, 5′-TAGAGAACCCACTGCTTACTGGC-3′, and a reverse primer 5′-CAGATGAACTTCAGGGTCAG-3′.

5′ RACE. 5′ RACE was performed using a 5′/3′ RACE kit (catalog no. 03353621001; Roche Applied Science) following the manufacturer's instruction. Briefly, Vero E6 cells seeded in six-well plates were transfected with 4 μg of either pCDNAGFP or pCDNAGFPns plasmid or cotransfected with 2 μg of each

GFP mRNA and nonsense GFP mRNA and synthesized by in vitro T7 transcription. Eight hours posttransfection, cells were infected with Sin Nombre virus at an MOI of 10. Cells were lysed 48 h postinfection, and total RNA was purified using the RNeasy kit (Qiagen). Two micrograms of purified total RNA was reverse transcribed using a primer, 5′-ACTAAAGCCAATCACACCCATGACA-3′, complementary to the S-segment mRNA from 696 to 720 nucleotides. The resulting cDNA was purified using a PCR cleanup kit (Qiagen), and a homopolymeric (dA) tail was added at the 3′ end of the cDNA, using terminal transferase provided in the kit. The (dA)-tailed cDNA was then used to generate a PCR product with the forward oligo(dT)-anchor primer 5′-GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTV-3′ (V = A, C, or T) and a reverse primer, 5′-GCGAAACTTAGAATGTAGAGTCCGATG-3′. The reverse primer was complementary to the S-segment mRNA from 405 to 431 nucleotides. Finally, the resulting PCR product was used as a template to generate a short PCR product using the anchor primer (5′-GACCACGCGTATCGATGTCGAC-3′) containing a MluI site and a reverse primer (5′-ATTATATAGCGGCCGCATGGTCATCAGGTTCAATCC-3′) containing a NotI site. The reverse primer was complementary to the S-segment mRNA from 290 to 309 nucleotides. The final PCR product was digested with MluI and NotI and cloned in pcDNA 3.1+ vector between the same restriction sites. The plasmid DNA isolated from 20 colonies was sequenced to read the cap-UTR junction of the S-segment mRNA.

Staining and microscopy. Adherent Huh-7 cells were grown on sterilized glass coverslips in a six-well plate. Cells were transfected with either GW182 siRNA (IDT) or Ge-1 siRNA (IDT) using Lipofectamine RNAiMax (Invitrogen) according to the manufacturer's instructions. Similarly, the control siRNA was transfected into the control well. After 24 h, cells were fixed at −20°C for 5 min using acetone and then permeabilized with 0.5% Triton X-100 at room temperature for 15 min. Cells were then incubated for 1 h at room temperature with rabbit anti-Dcp2 antibody at a dilution of 1:100 in phosphate-buffered saline (PBS) containing 2% fetal calf serum (FCS). After washing with PBS, cells were incubated for 1 h at room temperature with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG antibody at a 1,000-fold dilution in PBS containing 2% FCS. Fluorescent images were recorded by a Nikon Eclipse 80i upright microscope.

Synthesis of mRNA and 5′ capping. The mRNA synthesis was carried out using the Ribomax T7 transcription kit (Promega) as previously reported [37, 141, 146, 166]. Briefly, the gene of interest was PCR amplified using a forward primer containing a flanking T7 promoter and an appropriate reverse primer. The PCR product was gel purified and used as the template in a 50-µl transcription reaction. Following synthesis, template DNA was degraded with DNase I, and RNA was purified by RNAeasy (Qiagen) and stored in 10-µl aliquots at −70°C. The resulting mRNA was 5′ capped using the ScriptCap m7G capping system (Cell Script Catnumber C-SCCE0610), according to the manufacturer's instructions. Briefly, 50 µg of purified mRNA was added to the reaction mix containing 50 mM Tris HCl, pH 8.0, 6 mM KCl, 1.25 mM MgCl2, 1 mM GTP, 100

μM S-adenosyl-methionine (SAM), and 1 unit of the capping enzyme in a final volume of 100 μl. The reaction mixture was incubated at 37°C for 30 min, followed by purification of capped mRNA using the RNeasy kit (Qiagen). The purified capped mRNA was used to transfect Huh-7 cells.

Using this approach, we also synthesized GFP mRNA and nonsense GFP mRNA, which were cotransfected to Huh-7 cells to examine whether hantaviruses preferentially snatch caps from nonsense transcripts. Briefly, the GFP ORF was PCR amplified from pCDNAGFP plasmid using a forward primer, 5′-CTAGCTAATACGACTCACTATAGTAGAGAACCCACTGCTTACTGGCTTATCG-3′, and a reverse primer, 5′-CCATAGAGCCCACCGCATCCCC-3′. Similarly a forward primer, 5′-CTAGCTAATACGACTCACTATAGTAGAGAACCACCTGCTTACTGGCTTATCG-3′, and the above-described reverse primer were used to generate another PCR product from plasmid pCDNAGFPns. Both the PCR products were gel purified and used as the templates to generate two transcripts, as described above. The two transcripts were 5′ capped using the ScriptCap m7G capping system, as described above. The 3′ tailing of purified capped mRNAs was carried out using a Cell Script A-Plus poly(A) polymerase tailing kit, following the manufacturer's instructions. Briefly, 50 μg of purified capped GFP mRNA or nonsense GFP mRNA were mixed with 1× tailing buffer (50 mM Tris-HCl, pH 8.0, 250 mM NaCl, and 10 mM MgCl2) containing 10 mM ATP and 8 units of Cellscript A-plus poly(A) polymerase in a total volume of 50 μl. The reaction mixture was

incubated for 30 min at 37°C, and the tailed mRNA was purified by the RNeasy kit.

3. Results

SNV preferentially snatches caps from mRNAs containing premature termination codons. We have previously reported that hantavirus N protein efficiently protects the 5′ caps of cellular nonsense mRNAs in comparison to mRNAs, which encode proteins [37]. The protected caps were abundantly found in cellular P bodies, which were later efficiently used by the RdRp for transcription initiation [37]. To further confirm this observation, we cloned GFP in the pCDNA3.1+ vector, which expresses GFP mRNA having 5′ and 3′ UTRs of 71 and 372 nucleotides in length, respectively (Fig. 2E). In addition, we incorporated two extra nucleotides in the open reading frame (ORF) of the GFP expression construct, which generated a premature termination codon two amino acids downstream of the start codon (Fig. 2E). We transfected Vero E6 cells with these GFP constructs expressing either GFP mRNA or nonsense GFP mRNA, followed by infection with SNV 8 h posttransfection. We used our previously established cap-snatching assay to quantitatively estimate the caps snatched from either GFP mRNA or GFP nonsense mRNA by the viral RdRp. Briefly, 48 h postinfection, cells were lysed and total RNA was purified and reverse transcribed using a primer specific to the S-segment mRNA (Fig. 2A; also see Materials and Methods). The cDNA was PCR amplified using a forward primer specific to the 5′ terminus of GFP mRNA and a reverse primer specific to the N protein open reading frame (ORF) (Fig. 2A). This PCR strategy was designed to specifically identify the S-segment mRNAs, which have obtained their 5′ caps from either GFP mRNA or GFP nonsense mRNA. As expected, this PCR

strategy generated the PCR product of appropriate size only from SNV-infected cells that were previously transfected with the constructs expressing either GFP mRNA or GFP nonsense mRNA (Fig. 2B). A comparatively intense band from cells expressing GFP nonsense mRNA suggests that RdRp preferentially snatches caps from PTC-containing mRNAs. To rule out the possibility that the difference in the band intensities shown in Fig. 2B was not due to a difference in the intrinsic steady-state levels of GFP mRNA and GFP nonsense mRNA in host cells, we repeated the above-described experiment and quantitatively estimated the expression levels of these two transcripts using real-time PCR. As shown in Fig. 2C, the intrinsic steady-state levels of GFP mRNA were 3-fold higher than those of GFP nonsense mRNA, consistent with preferential degradation of PTC-containing mRNA by host NMD machinery. To further confirm the observation made in Fig. 2B that SNV RdRp preferentially snatches caps from PTC-containing mRNAs, we used our previously established real-time PCR-based cap-snatching method to quantitatively estimate the caps snatched by the S-segment mRNA from either GFP mRNA or GFP nonsense mRNA. Consistent with the observations made in Fig. 2C, we observed that although intrinsic steady-state levels of GFP nonsense mRNA were lower than those of GFP mRNA, the PTC-containing GFP mRNA served as a better cap donor than GFP mRNA (Fig. 2D).

In addition, we used 5′ RACE to examine the 5′ terminus of S-segment mRNA and to further confirm that SNV RdRp preferentially uses the PTC-containing mRNAs for cap snatching. We expressed either GFP mRNA or GFP nonsense

mRNA in virus-infected Vero E6 cells and examined the 5′ terminus of S-segment mRNA by 5′ RACE to further confirm that GFP nonsense mRNA serves as a preferential cap donor in comparison to GFP mRNA. The PCR product corresponding to virus-infected cells expressing either GFP mRNA or GFP nonsense mRNA was cloned, and plasmid DNA isolated from 20 colonies was sequenced (see Materials and Methods for details). Interestingly, 19 of 20 colonies were positive for cap snatching from GFP nonsense mRNA, suggesting that SNV RdRp snatched 95% of the caps from GFP nonsense mRNA in virus-infected cells expressing this transcript. In comparison, 1 of 20 colonies was positive for cap snatching from GFP mRNA, suggesting that only 5% of caps were snatched from GFP mRNA in virus-infected cells expressing this transcript (data not shown). Thus, both 5′ RACE and real-time PCR analysis suggest that the cap-donating potential of nonsense GFP mRNA is ~25-fold higher than that of GFP mRNA.

To further strengthen this observation, we synthesized the GFP mRNA and nonsense GFP mRNA by in vitro T7 transcription, as described in Materials and Methods. Both the mRNAs were capped at the 5′ terminus and polyadenylated at the 3′ terminus (see Materials and Methods). To differentiate the 5′ UTRs of GFP mRNA and nonsense GFP mRNA, we mutated the 10th and 11th residues downstream of the terminal cap in the 5′ terminus of GFP mRNA from CA to AC (Fig. 2F). We cotransfected Vero E6 cells with GFP mRNA and nonsense GFP mRNA, followed by viral infection. Total RNA was purified from infected cells, and 5′ RACE was again used to examine the 5′ terminus of viral S-segment mRNA,

as described above. An examination of 15 colonies revealed that 13 out of 15 colonies were positive for cap snatching from nonsense GFP mRNA. One colony each was positive for cap snatching from GFP mRNA and cellular mRNA. Taken together, these observations strongly establish that hantaviruses preferentially snatch caps from nonsense mRNAs.

It has been previously reported that hantavirus RdRp preferentially cleaves the host cell mRNA at a G residue during cap snatching[37, 69]. To confirm these hallmarks of correct cap snatching, the PCR products from Fig. 2B were cloned in a TA cloning vector (Invitrogen), and plasmid DNAs from 20 random clones were sequenced to examine the cap-UTR junction. Although the assay is not quantitative, it is evident from Fig. 2E that capped mRNAs are cleaved at G residues, with a preference for the 14th G residue downstream of the 5′ cap. In addition, this observation further confirms the specificity of the PCR-based cap-snatching assay. Similar observations were made from the 5′ RACE experiment (Fig. 2F).

SNV preferentially cleaves the host mRNA at a G residue 14 nucleotides downstream of the terminal cap. Since PTC-containing GFP mRNA was preferentially used for cap snatching, this mRNA was further examined to demonstrate the characteristics of the 5′ mRNA termini that are prerequisite for cap snatching. Both wild-type and PTC-containing GFP mRNAs contain five G residues in the first 30-nucleotide region of the 5′ UTR (Fig. 2E). Using site-directed mutagenesis, we first mutated these five G nucleotides to C residues

(pCDNAGFPnsG0) and asked whether the resulting mutant mRNA was still an efficient cap donor for the hantavirus cap snatching (Fig. 3A). We used a real-time PCR analysis to quantitatively estimate the caps obtained by the S-segment.

Sequence complementarity between the 3′ termini of the capped primer and viral genomic RNA favors cap snatching. To determine whether the complementarity between the nucleotides at the 3′ terminus of viral genomic RNA and the nucleotides located in the vicinity of the high-priority cleavage site (14th G residue) of the cap donor mRNA improves the efficiency for cap donation, we generated five additional mutants (Fig. 4A). The mutants pCDNAGFPns(i) and pCDNAGFPns(ii) express mRNAs having either two or three nucleotides at positions 13 to 14 or 12 to 14, respectively, complementary to the 3′ terminus of the viral genomic RNA (Fig. 4A). Similarly, three other mutants, pCDNAGFPns(iii), pCDNAGFPns(iv), and pCDNAGFPns(v), which have either three, six, or nine nucleotides complementary to the 3′ terminus of viral genomic RNA were generated (Fig. 4A). The mutant plasmids were transfected into Vero E6 cells, followed by viral infection, and caps obtained by the S-segment mRNA from the corresponding mutant mRNAs were quantified by real-time PCR, using the universal primer set. It is evident from Fig. 4B that a gradual increase in the nucleotide complementarity significantly enhances preferential usage of caps from the test mRNA during cap snatching. Although it has been previously reported that cleavage of capped mRNAs by the endonuclease subunit of

influenza virus RdRp occurs independent of the 3′ terminus of viral genomic RNA[173], the recent findings suggest that influenza virus transcriptase also prefers capped primers with 3′ nucleotides more complementary to the 3′ terminus of viral genomic RNA[63].

SNV snatches caps from its own mRNA. SNV preferentially snatched caps from the transcripts containing 5′ nucleotides complementary to the 3′ terminus of the viral genomic RNA (Fig. 4B). Caps were snatched with remarkable efficiency from the GFP nonsense transcript having a nine-nucleotide-long triplet repeat sequence (UAGUAGUAG) in the 5′ UTR, which is complementary to the 3′ terminus of viral genomic RNA (Fig. 4B). All hantaviral mRNAs contain this triplet repeat sequence at the same location, raising a question of whether hantaviruses snatch caps from their own mRNAs during infection. To test this hypothesis, we cloned the gene encoding the hantavirus glycoprotein precursor (GPC) along with 5′ and 3′ UTRs in the ptriEX1.1 vector. A PCR product was generated from the resulting plasmid using a forward primer containing a flanking 5′ T7 promoter preceded by the 14 nucleotides of the 5′ UTR of the GFP mRNA and a reverse primer containing a flanking 5′ poly (A) tail of 50 nucleotides in length. The resulting PCR was used as the template in a T7 transcription reaction for the synthesis of GPC mRNA (see Materials and Methods for details). The purified mRNA was capped at the 5′ terminus, as described in Materials and Methods. This capping method incorporates cap 1 structure, predominantly found in all higher eukaryotic mRNA[174]. The cap 1 structure was found to be required for

priming the transcription of influenza virus mRNA[62]. The synthesis of GPC mRNA was performed in such a way that the 5′ 14 nucleotides of the GPC transcript matched all other transcripts used in this study (Fig. 5A). In addition, the synthesized GPC mRNA also matched the transcript generated by the viral RdRp. Similarly; we synthesized the GPCns mRNA containing a PTC 15 amino acids downstream of the initiating methionine (Fig. 5A). For comparison, we also synthesized GFP mRNA and GFP nonsense mRNA using T7 RNA polymerase (Fig. 5A). Vero E6 cells were transfected with these mRNAs, and the expression of GPC and GFP was monitored by Western blot analysis. Unlike nonsense mRNAs, the GFP and GPC expression was observed in cells transfected with GFP mRNA and GPC mRNA, respectively (Fig. 5B, inset). The cap-donating potential of these four transcripts (Fig. 5A) was quantified by a real-time PCR, using the primer set shown in Fig. 2A. As shown in Fig. 5B, SNV snatched caps from both mRNAs encoding either GFP or viral GPC protein. However, both GFP and GPC mRNAs harboring PTCs served as efficient cap donors in comparison to their respective wild-type mRNAs. Moreover, it is noticeable from Fig. 5B that in comparison to GFP nonsense mRNA, SNV preferentially snatched caps from its own mRNA having a PTC.

Since GPC generates two transmembrane proteins, Gn and Gc, the translation of GPC mRNA occurs on the endoplasmic reticulum (ER). The GFP is a soluble cytoplasmic protein; it is likely that GFP mRNA is translated by the cytoplasmic ribosomes. Thus, the possibility that hantaviruses preferentially snatch caps from nonsense mRNAs associated with ER cannot be ruled out.

Role of cellular P bodies in hantavirus cap snatching. We have previously reported that capped host mRNA oligoribonucleotides bound with SNV N protein were abundantly found in cellular P bodies that were later efficiently used as primers by the hantavirus RdRp for transcription initiation (42). It is possible that N independently migrates to P bodies and selectively associates with the 5′ caps of those cellular transcripts that are transported to P bodies for degradation. Alternatively, it is equally likely that N independently associates with the mRNA 5′ caps in the cytoplasm and migrates to P bodies along with bound mRNAs, which are targeted to P bodies for degradation. To test these two possibilities and to demonstrate the general role of cellular P bodies in hantavirus cap snatching, we used siRNAs to downregulate GW182 and Ge-1 proteins in Huh-7 cells. Downregulation of Gw182 and Ge-1 was confirmed by Western blot analysis (Fig. 6A and B). Both Gw182 and Ge-1 are critical P-body components whose downregulation has been reported to cause P-body loss in cells [98, 175]. The decapping enzyme Dcp2 is a P-body resident and a commonly used marker to examine the P-body formation in cells. Using an anti-Dcp2 antibody, the P-body formation in wild-type and siRNA-downregulated Huh-7 cells was examined by fluorescence microscopy. As evident from Fig. 6C, the downregulation of GW182 and Ge-1 causes P-body loss in Huh-7 cells.

We next asked whether SNV N can bind to the 5′ caps and equally protect the 5′ terminus of the nonsense mRNA in wild-type and P-body-deficient Huh-7 cells. If N selectively protects the 5′ caps of cellular mRNAs in P bodies, we do not

expect such protection in Gw182-deficient cells. We cotransfected the wild-type and P-body-deficient Huh-7 cells with a pCDNAGFPns construct expressing nonsense GFP mRNA along with a plasmid expressing SNV N protein. At 36 h posttransfection, cells were lysed and total RNA was purified and reverse transcribed using random primers, as previously reported [37]. The 5′ terminus of GFP nonsense mRNA was quantitatively estimated by real-time PCR analysis using a primer set targeted to amplify 180 nucleotides at the 5′ terminus of the mRNA, as previously reported [37]. As shown in Fig. 6D, the loss of P-body machinery resulted in the protection of the 5′ mRNA terminus independent of N protein expression. This protection was likely due to the inefficient decapping machinery in P-body-downregulated cells [98, 175]. Interestingly, we observed a remarkable protection of the 5′ mRNA terminus by SNV N in both wild-type and P-body-downregulated cells. This observation suggests that SNV N likely binds to the capped host cell mRNAs in the cytoplasm outside the P bodies. To delineate whether P bodies play a role in hantavirus cap snatching and viral mRNA synthesis, we transfected the Huh-7 cells with the cap donor plasmid pCDNAGFPns, followed by the viral infection. The cap-snatching efficiency by SNV RdRp in P-body-deficient Huh-7 cells was compared with that of wild-type Huh-7 cells having intact P-body machinery. Since the protection of the 5′ mRNA terminus by N protein in P-body-deficient cells was around 2-fold greater than that in the wild-type Huh-7 cells (Fig. 6D), we expected corresponding increases in cap snatching in P-body-deficient cells. Interestingly, we observed that cap snatching, and hence viral mRNA synthesis, was not improved in P-body-

deficient cells compared to that in wild-type cells (Fig. 6E). This observation suggests that N increases the RNA half-life with no mechanism inferred.

4. Discussion

The viral genome of negative-sense RNA viruses is encapsidated into viral nucleocapsids, which serve as templates for RdRp during transcription and replication of the viral genome[176, 177]. The negative-strand segmented RNA viruses of the Orthomyxoviridae (e.g., influenza A, B, and C and Thogoto viruses), Bunyaviridae (e.g., La Crosse, hanta, Rift Valley fever, and Crimean-Congo hemorrhagic fever viruses) and Arenaviridae (e.g., Lassa virus) families synthesize the translation-competent capped mRNAs by the cap-snatching mechanism. This mechanism has been well studied for the influenza virus, having an RdRp composed of three subunits, PA, PB1, and PB2. The PB1 subunit contains a conserved polymerase domain, which carries out RNA elongation during RNA synthesis[152]. The PB2 subunit binds to the 5′ caps of host pre-mRNAs[64, 66], and the PA subunit has the endonuclease domain, which cleaves the capped pre-mRNAs 10 to 13 nucleotides downstream of the 5′ cap[67, 153]. The capped oligoribonucleotides are used as primers by the viral RdRp to initiate the transcription. The presence of an endonuclease domain in the RdRps of several other viruses, including SNV, has recently been proposed[52, 163]. However, it is not yet clear whether, similarly to that in influenza viruses, the entire cap-snatching process in these viruses is carried out solely by the RdRp or other host factors or whether viral proteins also play a role. Sequence analyses of many viral mRNA 5′ termini have revealed a nucleotide preference at the 3′ end of the capped primer, which has been assumed to reflect the sequence preference for cleavage by the viral endonuclease during cap

snatching. For example, in the case of Dugbe virus endonucleolytic cleavage is assumed to take place after a C residue[159], whereas for Bunyamwera virus a strong preference for cleavage after a U residue has been proposed[51]. In tomato spotted wilt virus, preference for an A residue has been confirmed[178]. However, it has been reported that influenza virus RdRp effectively uses only CA-terminated capped fragments as primers for viral mRNA synthesis *in vitro*. Consistent with our previous observation[37], we have found that primers used by the SNV RdRp are terminated at G residues. Taking these observations into consideration, it is likely that RdRps have a preference for the endonucleolytic cleavage at certain nucleotides in the mRNA sequence. However, it is also possible that RdRps randomly cleave the capped mRNAs and that the resulting capped fragments with appropriate terminal nucleotides are selected as primers. The selection may depend upon the appropriate location of complementary nucleotides at the 3′ terminus of the vRNA template.

Previous studies have reported that most viruses use 15-nucleotide-long capped primers with a variation of 10 to 20 nucleotides for transcription initiation [133, 150, 151, 154, 157-161]. However, viruses of the Arenaviridae and the nairovirus genus use relatively shorter primers, varying in length from 1 to 4 and 5 to 16 nucleotides, respectively[69, 156, 159]. We observed that SNV has a strong preference for 14-nucleotide-long primers containing a 3′-terminal G residue. It is still a mystery why the RdRps from different viruses use capped primers of various lengths for the transcription initiation. A possible role of the length of a

capped primer in transcription initiation has been suggested in the cap-snatching model (Fig. 7).

In hantaviruses, the capped primer containing a 3′ G residue has been proposed to undergo base pairing with one of the C residues at the 3′ terminus of the vRNA template during transcription initiation[69]. It has been suggested that RdRp elongates the annealed primer during transcription initiation using a “prime and realign” mechanism[37, 69]. However, it is interesting to imagine how a single G-C base pairing between the RNA primer and 3′ terminus of the vRNA template stabilizes the primer and favors its annealing. We have previously reported that hantavirus N protein stabilizes a capped primer at the 3′ terminus of the vRNA template[32]. We showed that N protein binds the mRNA caps and has distinct cap and RNA binding sites[36, 37]. N protein with a capped primer loaded at its cap-binding site simultaneously binds the 3′ terminus of the vRNA template with specificity and facilitates the annealing of the capped primer with the template, which favors transcription. To further address the annealing of the capped primer with the vRNA template during the cap-snatching mechanism of transcription initiation, we asked whether the complementarity between the 3′ termini of the primer and vRNA template favors cap snatching. We incorporated two nucleotides preceding the high-priority cleavage site (14th G) in the 3′ terminus of test mRNA which were complementary to the 3′ terminus of the genomic RNA (Fig. 4A). Similarly, either three, six, or nine nucleotides complementary to the 3′ terminus of the vRNA template were incorporated in the test mRNA, preceding the 14th G residue (Fig. 4A). These complementary nucleotides contained G

residues, which can also serve as low-priority cleavage sites for the RdRp. An examination by a very sensitive real-time PCR method demonstrated that these test mRNAs served as efficient cap donors during cap snatching. These observations support the recent findings that although the cleavage of capped mRNAs by the endonuclease subunit of influenza RdRp occurs independent of the 3′ terminus of viral genomic RNA[173], the influenza virus transcriptase prefers capped primers with 3′ nucleotides more complementary to the 3′ terminus of viral genomic RNA[63]. We propose that for an mRNA to be an efficient cap donor during cap snatching, it must contain a high-priority cleavage site at an appropriate length from the 5′ terminus. For example, an mRNA having a G residue at the 14th position will be the preferred cap donor for hantavirus cap snatching. In addition, the nucleotide complementarity between the 3′ termini of the capped primer and vRNA template favors the annealing of the primer with the vRNA template.

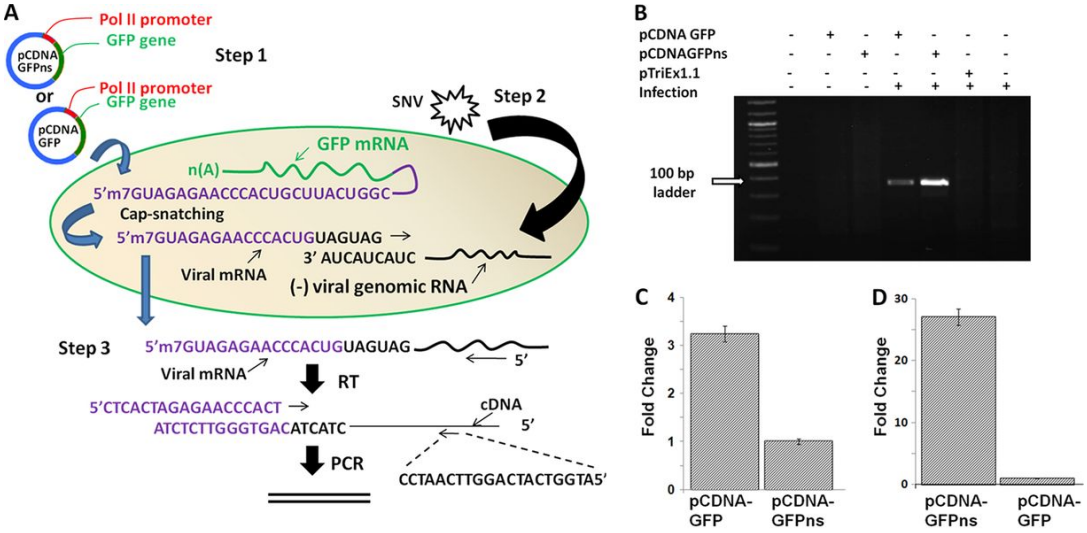
Although the basic mechanism for the generation of capped primers of appropriate length and specificity might be similar between the RdRps of influenza virus and SNV, the basic difference in their cap-snatching mechanism would be due to their replications at different locations in the host cell. The cellular mRNAs are engaged in translation in the cell cytoplasm and are targeted to P bodies for degradation after the completion of translation. Thus, unlike influenza virus, whose replication takes place in the nucleus, the viruses replicating in the cytoplasm, such as SNV and Rift Valley Fever virus, have to effectively compete with the host decapping machinery to protect the mRNA caps

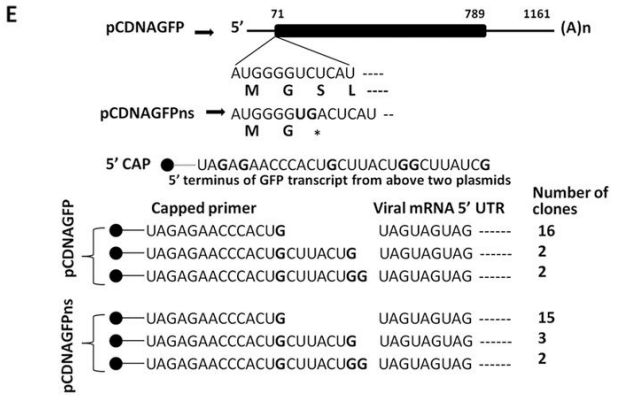
from degradation. We have previously reported that hantavirus N protein actively protects the host mRNA caps from degradation[37]. N binds to the host mRNA caps and likely blocks the binding of the decapping enzyme DCP2 at the mRNA 5′ cap by competitive inhibition, which would result in the protection of host mRNA caps from degradation. The rescued 5′ capped RNA oligoribonucleotides were sequestered in the cellular P bodies by N and were later efficiently used as primers by the RdRp. These observations raised the questions of whether N binds to the mRNA caps in the cytoplasm or inside the P bodies and whether P bodies have any role in the cap-snatching process. Protection of host mRNA caps in P-body-downregulated cells support the idea that N likely binds the mRNA caps inside the cytoplasm. N protein is likely transported to the P bodies along with the mRNAs, which are targeted for degradation after the completion of translation. Although N rescued the mRNA caps in P-body-deficient cells, the rescued caps were not used with significant efficiency in comparison to cells having intact P-body machinery. These observations suggest that P bodies might play a role in virus replication by providing the capped primers with high efficiency for the transcription initiation. Alternatively, a possible role of a P-body component in hantavirus cap snatching cannot be ruled out. Further experimentation is required to demonstrate the exact role of cellular P bodies in the cap-snatching process. A critical observation that PTC-containing mRNAs were remarkably efficient cap donors in comparison to the wild-type mRNAs suggests that efficiency of an mRNA to donate caps for viral mRNA synthesis is regulated primarily at the translation process. An mRNA engaged in translation

may not be an efficient cap donor even if it contains a potential G residue at the 14th position or contains a sequence more complementary to the 3′ terminus of the viral RNA template. We propose that hantaviruses obtain most of their caps from the mRNAs, which abort translation due to PTCs or other translation defects.

The interesting observation that hantaviruses can snatch caps from their own mRNAs is consistent with the positive role of the nucleotide complementarity between the 3′ termini of the capped primer and the vRNA template in cap snatching. However, the cap-donating efficiency of wild-type mRNAs is negligible in comparison to that of the nonsense mRNAs; it is unlikely that viral mRNAs will be efficiently used for cap snatching during viral infection due to their continued engagement in the translation. We found that viral mRNAs containing a PTC were remarkably used for cap snatching with high efficiency. Hantavirus RdRp lacks proofreading activity. The promiscuous nature of polymerization by RdRps due to the lack of proofreading ability is thought to be the primary source of evolution in RNA viruses, which provides them an ability to replicate in different hosts and produce more pathogenic strains. An error rate of approximately 1 mutation/replication/genome for hantaviruses has been estimated. Although this mutation rate is negligible, the nonsense mRNAs generated due to the lack of proofreading activity of the RdRp will be actively recycled for cap snatching. We suggest that apart from helping in the evolutionary strategies of the virus, the lack of proofreading activity of the RdRp plays a role in cap snatching. A model

depicting the role of N protein and cellular P bodies in hantavirus cap snatching is shown in Fig. 7.





F

GFP mRNA → m7G-TAGAGAACCCACUGC—GFP—(A)n

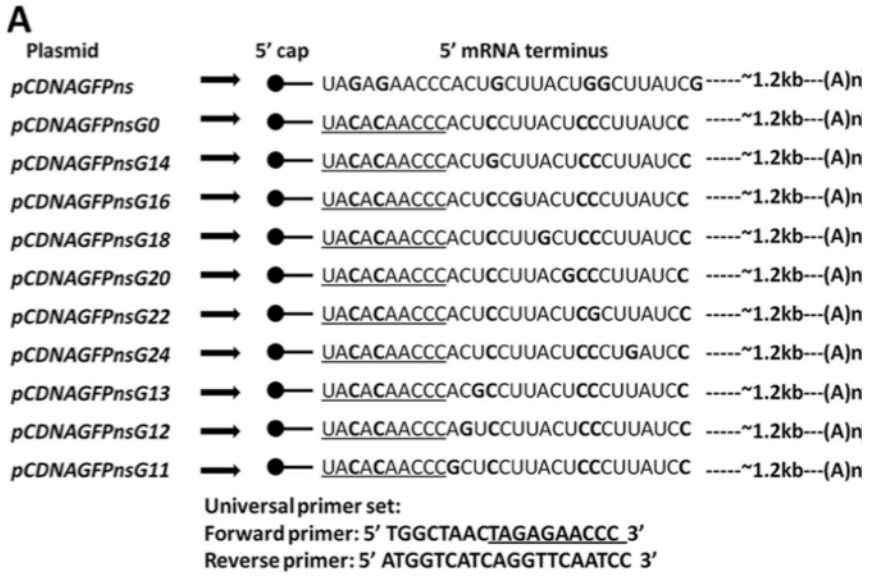
Nonsense GFP mRNA → m7G-TAGAGAACCACCUGC—GFP—(A)n

Capped primer	Snatched from	Viral mRNA 5' UTR	# clones
5'-UAGAGAACCACCUG	Non sense GFP mRNA	*UAGUAGUAG*	*13*
5'-UAGAGAACCCACUG	GFP mRNA	*UAGUAGUAG*	*1*
5' AUAGACAUUGTTGG	Cellular	*UAGUAGUAG*	*1*

Figure 2 Hantavirus cap-snatching assay.

(A) A diagrammatic representation of the cap-snatching assay. Step 1, Vero E6 cells were transfected with cap donor plasmid (pCDNAGFPns or pCDNAGFP) followed by SNV infection 4 h posttransfection (step 2). Step 3, cells were lysed 48 h postinfection, and total RNA was purified as described in Materials and Methods. Twenty-five nanograms of the purified RNA was reverse transcribed using a primer specific to the S-segment-derived mRNA. The cDNA was PCR amplified using a forward primer specific to the 5′ terminus of GFP mRNA (purple) and a reverse primer specific to the N gene (black). See Materials and Methods for details. (B) As expected, the PCR product was generated only from the cells which were transfected with either pCDNAGFPns or pCDNAGFP plasmid, followed by viral infection. (C) The Vero E6 cells were transfected with cap donor plasmid pCDNAGFPns or pCDNAGFP, followed by SNV infection 4 h posttransfection, as described for panel A. Cells were lysed 48 h postinfection, and total RNA was purified. Intrinsic mRNA levels expressed from cap donor plasmids pCDNAGFPns and pCDNAGFP were quantified by real-time PCR analysis using a primer set specific to the GFP open reading frame, as described in Materials and Methods. (D) Similarly, the cap-donating potential of the transcripts expressed from cap donor plasmids pCDNAGFPns and pCDNAGFP was determined by real-time PCR using a primer set shown in panel A. (E) The PCR product from panel B was cloned in a TA cloning vector (Invitrogen), and plasmid DNA from 20 random clones was sequenced to examine the cap-UTR junction. As shown at the bottom, the capped primers were terminated at the 3′ G

residue. (F) The GFP mRNA and nonsense GFP mRNA were synthesized by in vitro T7 transcription, as described in Materials and Methods. To distinguish their 5′ UTRs, two nucleotides in the 5′ UTR of nonsense GFP mRNA were mutated (bold and underlined). Vero E6 cells were cotransfected with GFP mRNA and nonsense GFP mRNA, followed by virus infection. The cap-donating potential of these two transcripts was examined by 5′RACE, as described in Materials and Methods.


A
Plasmid
5' cap
5' mRNA terminus
pCDNAGFPns
UAGAGAACCCACUGCUUACUGGCUUAUCG-----~1.2kb---(A)n
pCDNAGFPnsG0
UACACAACCCACUCCUUACUCCCUUAUCC-----~1.2kb---(A)n
pCDNAGFPnsG14
UACACAACCCACUGCUUACUCCCUUAUCC-----~1.2kb---(A)n
pCDNAGFPnsG16
UACACAACCCACUCCGUACUCCCUUAUCC-----~1.2kb---(A)n
pCDNAGFPnsG18
UACACAACCCACUCCUUGCUCCCUUAUCC-----~1.2kb---(A)n
pCDNAGFPnsG20
UACACAACCCACUCCUUACGCCCUUAUCC-----~1.2kb---(A)n
pCDNAGFPnsG22
UACACAACCCACUCCUUACUCGCUUAUCC-----~1.2kb---(A)n
pCDNAGFPnsG24
UACACAACCCACUCCUUACUCCCUGAUCC-----~1.2kb---(A)n
pCDNAGFPnsG13
UACACAACCCACGCCUUACUCCCUUAUCC-----~1.2kb---(A)n
pCDNAGFPnsG12
UACACAACCCAGUCCUUACUCCCUUAUCC-----~1.2kb---(A)n
pCDNAGFPnsG11
UACACAACCCGCUCCUUACUCCCUUAUCC-----~1.2kb---(A)n
Universal primer set:
Forward primer: 5' TGGCTAACTAGAGAACCC 3'
Reverse primer: 5' ATGGTCATCAGGTTCAATCC 3'

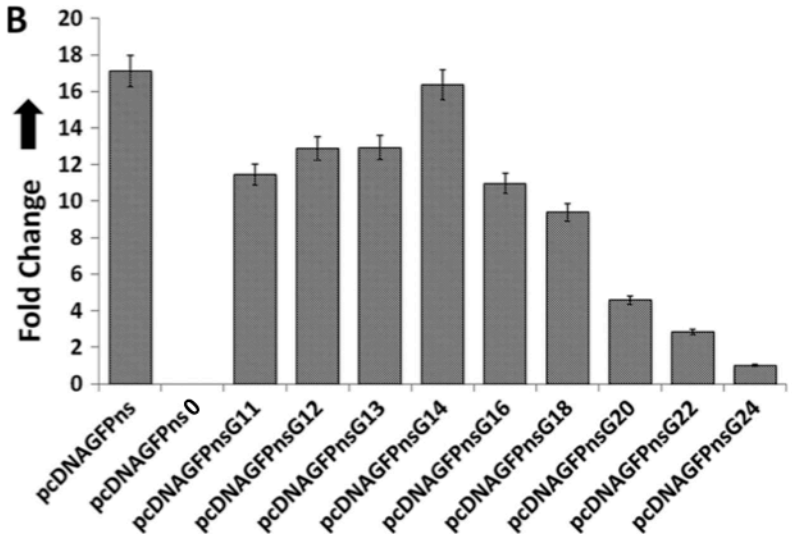

B
Fold Change
0
2
4
6
8
10
12
14
16
18
20
pcDNAGFPns
pcDNAGFPns 0
pcDNAGFPnsG11
pcDNAGFPnsG12
pcDNAGFPnsG13
pcDNAGFPnsG14
pcDNAGFPnsG16
pcDNAGFPnsG18
pcDNAGFPnsG20
pcDNAGFPnsG22
pcDNAGFPnsG24

Figure 3 Characteristics of a preferred cap donor mRNA for hantavirus cap snatching.

(A) To determine the characteristics of the 5′ mRNA termini that are required for cap snatching, we generated multiple constructs, shown on the left side. The mRNA encoded by each plasmid is shown by an arrow. The bold letters show the mutations in the 5′ mRNA terminus. The universal primer set used in the real-time PCR studies is shown at the bottom. (B) Vero E6 cells were transfected with the plasmids shown in panel A, followed by SNV infection. Total mRNA was purified and reverse transcribed using a primer specific to the S-segment mRNA. Using the universal primer set, the cDNA was used in real-time PCR analysis to quantify the caps obtained by the S-segment mRNA from the mutant mRNAs expressed from the transfected plasmid of interest (see Materials and Methods for details).

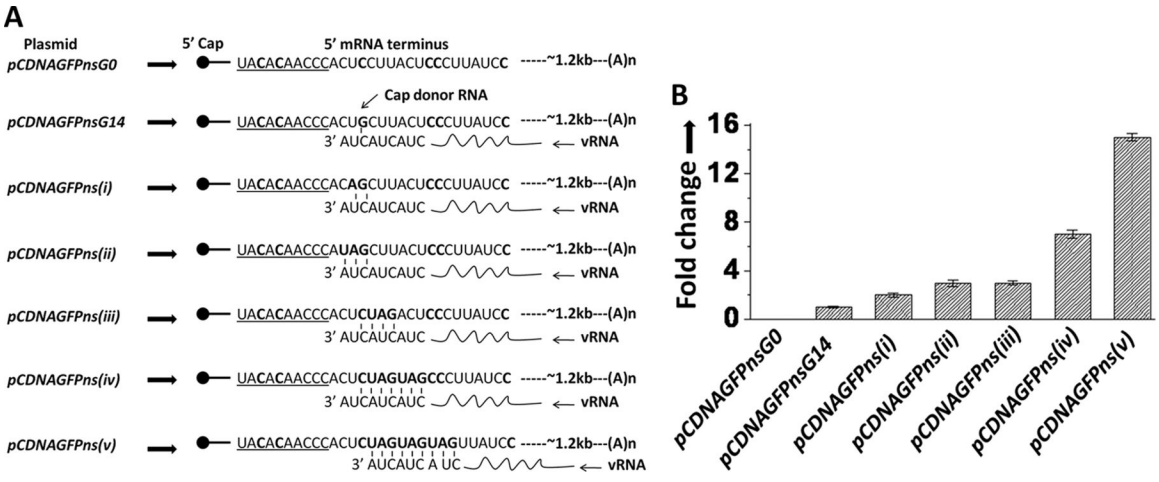

A
Plasmid
5′ Cap
5′ mRNA terminus
pCDNAGFPnsG0
UACACAACCCACUCCUUACUCCCCUUAUCC -----~1.2kb---(A)n
Cap donor RNA
pCDNAGFPnsG14
UACACAACCCACUGCUUACUCCCCUUAUCC -----~1.2kb---(A)n
3′ AUCAUCAUC ← vRNA
pCDNAGFPns(i)
UACACAACCCACAGCUUACUCCCCUUAUCC -----~1.2kb---(A)n
3′ AUCAUCAUC ← vRNA
pCDNAGFPns(ii)
UACACAACCCAUAGCUUACUCCCCUUAUCC -----~1.2kb---(A)n
3′ AUCAUCAUC ← vRNA
pCDNAGFPns(iii)
UACACAACCCACUCUAGACUCCCCUUAUCC -----~1.2kb---(A)n
3′ AUCAUCAUC ← vRNA
pCDNAGFPns(iv)
UACACAACCCACUCUAGUAGCCCCUUAUCC -----~1.2kb---(A)n
3′ AUCAUCAUC ← vRNA
pCDNAGFPns(v)
UACACAACCCACUCUAGUAGUAGUUAUCC -----~1.2kb---(A)n
3′ AUCAUC A UC ← vRNA
B
Fold change
16
12
8
4
0
pCDNAGFPnsG0
pCDNAGFPnsG14
pCDNAGFPns(i)
pCDNAGFPns(ii)
pCDNAGFPns(iii)
pCDNAGFPns(iv)
pCDNAGFPns(v)

Figure 4 Role of nucleotide complementarity in hantavirus cap snatching. (A) To determine whether nucleotide complementarity at the 3′ termini of viral genomic RNA and the capped primer have a role in cap snatching, we generated five additional plasmids, shown on the left side. The mRNAs expressed from these plasmids are shown by arrows. The bold letters show the mutations in the 5′ mRNA terminus. The complementary nucleotides between the test mRNA and the viral genomic RNA are shown. (B) Vero E6 cells were transfected with the plasmids shown in panel A, followed by SNV infection. Total mRNA was purified and reverse transcribed using a primer specific to the S-segment mRNA. Using the universal primer set, the cDNA was used in real-time PCR analysis to quantify the caps obtained by the S-segment mRNA from the mutant mRNAs expressed from the transfected plasmids shown in panel A (see Materials and Methods for details).

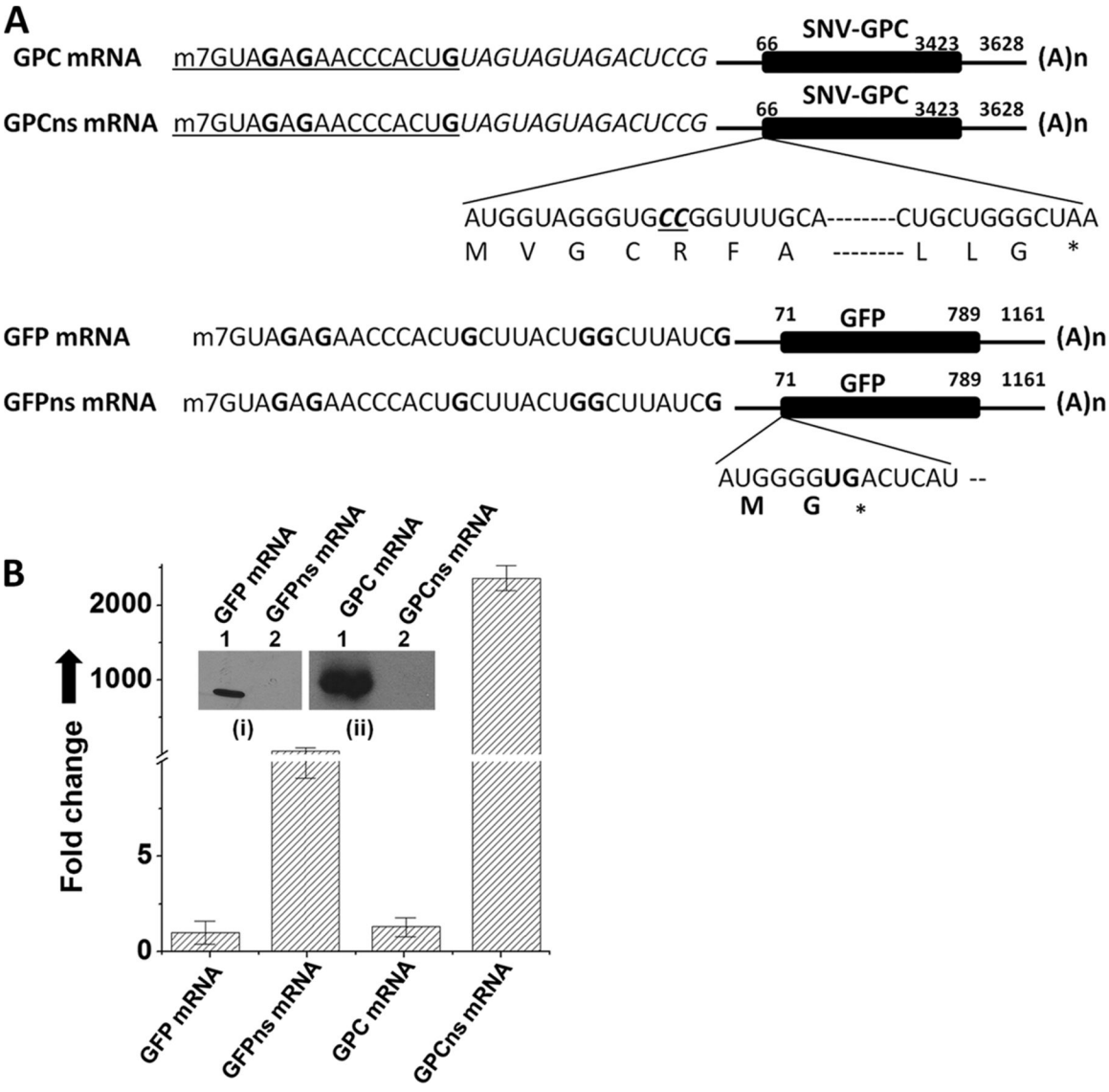

A
GPC mRNA
m7GUAGAGAACCCACUGUAGUAGUAGACUCCG
66
SNV-GPC
3423
3628
(A)n
GPCns mRNA
m7GUAGAGAACCCACUGUAGUAGUAGACUCCG
66
SNV-GPC
3423
3628
(A)n
AUGGUAGGGUGCCGGUUUGCA--------CUGCUGGGCUAA
M V G C R F A -------- L L G *
GFP mRNA
m7GUAGAGAACCCACUGCUUACUGGCUUAUCG
71
GFP
789
1161
(A)n
GFPns mRNA
m7GUAGAGAACCCACUGCUUACUGGCUUAUCG
71
GFP
789
1161
(A)n
AUGGGGUGACUCAU --
M G *
B
GFP mRNA
GFPns mRNA
GPC mRNA
GPCns mRNA
1
2
1
2
(i)
(ii)
Fold change
2000
1000
5
0
GFP mRNA
GFPns mRNA
GPC mRNA
GPCns mRNA

Figure 5 SNV snatches caps from its own mRNA.

(A) To determine whether hantaviruses snatch caps form their own mRNAs, we synthesized SNV glycoprotein precursor (GPC) mRNA and GPC nonsense mRNA by T7 transcription reaction, as described in Materials and Methods. The two mRNAs are the same in sequence except that nonsense GPC mRNA has two additional CC residues in the GPC ORF four amino acids from the initiating methionine. This additional mutation generated a stop codon 15 amino acids downstream of the initiating methionine. For comparison we synthesized GFP mRNA and nonsense GFP mRNA using a T7 transcription reaction, as described in Materials and Methods. GFP mRNA and nonsense GFP mRNA are the same in sequence except that nonsense GFP mRNA has a PTC two amino acids from the initiating methionine. It must be noted that the 5′-terminal 15 nucleotides of all these four mRNAs are the same in sequence. (B) Vero E6 cells were transfected with the mRNAs shown in panel A, followed by SNV infection. Caps snatched by SNV RdRp from these four transcripts were quantified by real-time PCR analysis using the universal primer set shown in Fig. 3A (see Materials and Methods for details). To ensure that transfected mRNA were properly engaged in translation, Vero E6 cells were transfected with the mRNA shown in panel A. Cells were lysed 36 h posttransfection, and cell lysates were examined by Western blot analysis (inset) using either anti-GFP (i) or anti-His tag (ii) antibody. The GPC contained a C-terminal His tag and was examined by ant-His tag antibody.

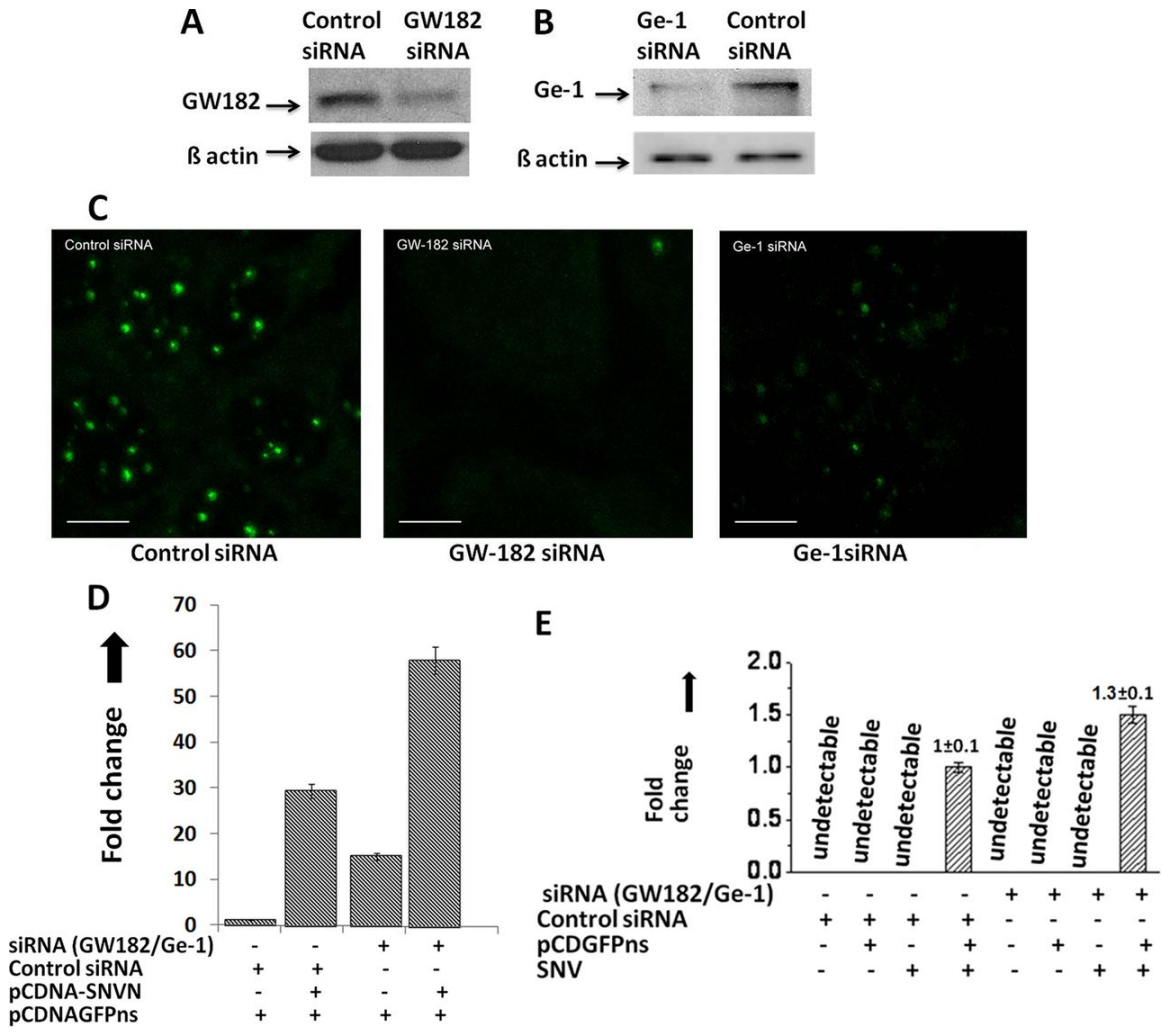

A
Control siRNA
GW182 siRNA
GW182
ß actin
B
Ge-1 siRNA
Control siRNA
Ge-1
ß actin
C
Control siRNA
GW-182 siRNA
Ge-1 siRNA
Control siRNA
GW-182 siRNA
Ge-1siRNA
D
Fold change
70
60
50
40
30
20
10
0
siRNA (GW182/Ge-1) - - + +
Control siRNA + + - -
pCDNA-SNVN - + - +
pCDNAGFPns + + + +
E
Fold change
2.0
1.5
1.0
0.5
0.0
undetectable
undetectable
undetectable
1±0.1
undetectable
undetectable
undetectable
1.3±0.1
siRNA (GW182/Ge-1) - - - - + + + +
Control siRNA + + + + - - - -
pCDGFPns - + - + - + - +
SNV - - + + - - + +

Figure 6 Role of cellular P bodies in hantavirus cap snatching.

(A) Downregulation of GW182 by siRNA. To determine whether GW182 was downregulated after siRNA treatment, cells transfected with either siRNA or control siRNA were lysed and GW182 expression was monitored by Western blot analysis using anti-GW182 antibody. To confirm that downregulation of GW182 observed by Western blot analysis was not a loading error, equal volumes of cell lysate were examined for the expression of β-actin by Western blot analysis (bottom bands). (B) Similar to what is shown in panel A, the downregulation of Ge-1 by siRNA was confirmed by Western blot analysis using anti-Ge-1 antibody. (C) Downregulation of GW182 and Ge-1 by siRNA causes the downregulation of cellular P bodies. Huh-7 cells were transfected with 150 nM either control siRNA, GW-182 siRNA, or Ge-1 siRNA. Twenty-four hours posttransfection, cells were fixed with formaldehyde and stained with anti-Dcp2 antibody. Cells were visualized under fluorescence microscope using a FITC-conjugated secondary antibody. Bar, 10 μm. (D) Wild-type or P-body-downregulated Huh-7 cells were cotransfected with pCDNAGFPns and pCDNA-SNVN plasmids, expressing the cap donor test mRNA and SNV N protein, respectively. Thirty-six hours posttransfection, cells were lysed and total RNA was purified and reverse transcribed using random primers. The effect of N upon the stability of the test mRNA was quantified by real-time PCR analysis using a forward primer, 5′-TAGAGAACCCACTGCTTACTGGC-3′, and a reverse primer, 5′-CAGATGAACTTCAGGGTCAG-3′, to amplify 241 nucleotides from the 5′ mRNA terminus. (E) Huh-7 cells transfected with either siRNA or control siRNA along

the cap donor plasmid pCDNAGFPns were infected with SNV. Caps snatched by the SNV from the test mRNA were quantified by real-time PCR analysis using the primer set shown in Fig. 2A.

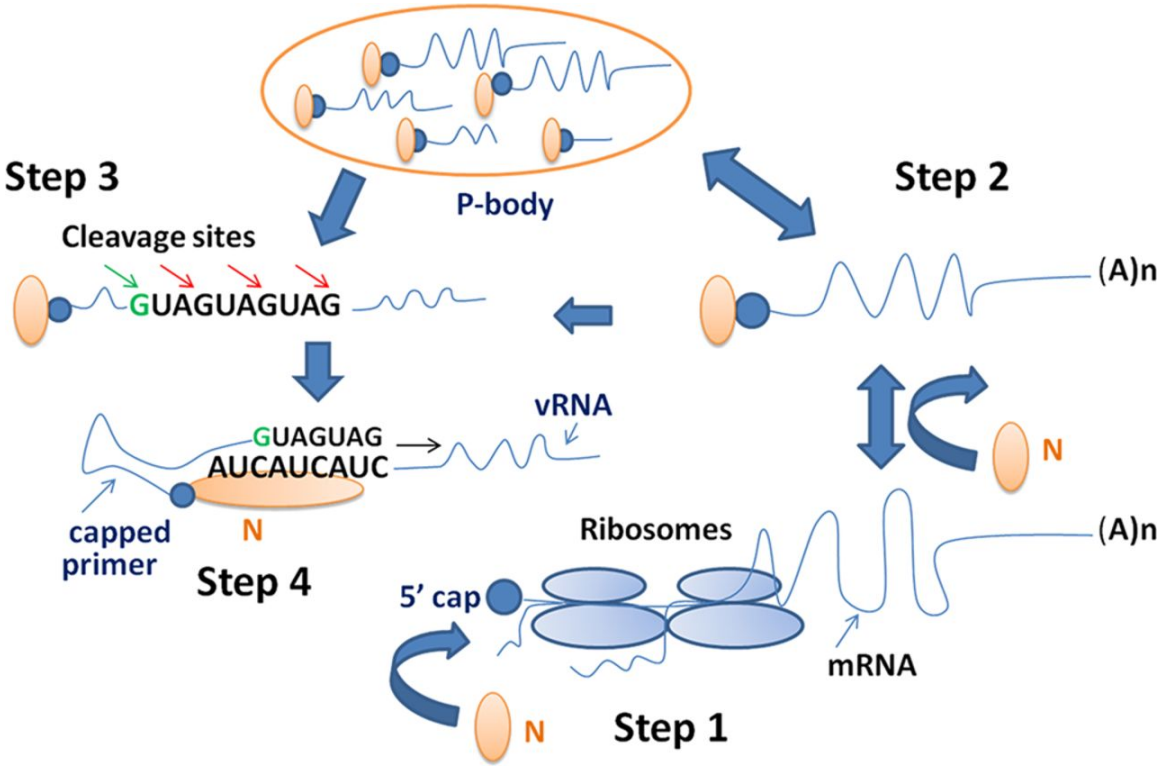

P-body
Step 3
Step 2
Cleavage sites
GUAGUAGUAG
(A)n
vRNA
GUAGUAG
AUCAUCAUC
N
capped
primer
N
Step 4
Ribosomes
(A)n
5' cap
mRNA
N
Step 1

Figure 7 Hantavirus cap-snatching model.

(Step 1) An mRNA engaged in translation is not used for cap snatching. N protein can bind to the mRNA cap before translation or after the completion of translation. (Step 2) N-associated mRNAs are targeted to P bodies for degradation. N protects the mRNA caps from degradation in P bodies. The rescued 5′-capped mRNA oligoribonucleotides are stored in P bodies by N and are efficiently used for cap snatching outside P bodies. Alternatively, N-associated mRNAs outside the P bodies can be directly used in cap snatching with less efficiency. (Step 3) The endonuclease activity of hantavirus RdRp or a possible cellular endonuclease preferentially cleaves the capped oligonucleotides at G residues. (Step 4) N protein with a capped primer at the cap binding site simultaneously binds the 3′ terminus of the viral RNA genome and facilitates the annealing of the capped primer with the 3′ terminus of the vRNA template. The capped primer with 3′ nucleotides more complementary to the 3′ terminus of vRNA genome is preferred for transcription initiation.

Chapter III

Interaction between Hantavirus Nucleocapsid Protein (N) and RNA-Dependent RNA Polymerase (RdRp) Mutants Reveals the Requirement of an N-RdRp Interaction for Viral RNA Synthesis

Abstract

Viral ribonucleocapsids harboring the viral genomic RNA are used as the template for viral mRNA synthesis and replication of the viral genome by viral RNA-dependent RNA polymerase (RdRp). Here we show that hantavirus nucleocapsid protein (N protein) interacts with RdRp in virus-infected cells. We mapped the RdRp binding domain at the N terminus of N protein. Similarly, the N protein binding pocket is located at the C terminus of RdRp. We demonstrate that an N protein-RdRp interaction is required for RdRp function during the course of virus infection in the host cell.

Results and Discussion

Hantaviruses are negative-strand emerging RNA viruses and members of the Bunyaviridae family. Humans get hantavirus infection by inhalation of aerosolized excreta from infected rodent hosts[179-182]. Hantavirus infection causes hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS), with mortalities of 15% and 50%, respectively[130, 131]. The spherical hantavirus particles harbor three genomic RNA segments (S, M, and L) within a lipid bilayer [131, 183], encoding viral nucleocapsid protein (N protein), viral RNA-dependent RNA polymerase (RdRp), and glycoprotein precursor (GPC), respectively. The GPC is posttranslationally cleaved at a highly conserved WAASA motif, generating two glycoproteins, Gn and Gc[184]. N protein is multifunctional, primarily involved in encapsidation of the viral genome. However, recent studies have suggested that N protein plays diverse roles in the virus replication cycle. The viral ribonucleocapsids are used as the template by viral RdRp for the synthesis of viral mRNA and replication of the viral genome [176, 177]. However, it is still a mystery how RdRp accesses the viral RNA (vRNA) genome, which is buried inside the compact nucleocapsid structure. Moreover, numerous reverse genetic systems have proven that RdRp from negative-strand RNA viruses requires assistance from N protein while performing its function inside the host cell[139, 185-188]. Based on these observations, we asked whether N protein directly interacts with RdRp and regulates its function.

To answer this question, we cloned the gene encoding the Sin Nombre virus (SNV) RdRp in the pcDNA 3.1 (+) backbone containing either a green fluorescent protein (GFP) tag at the N terminus or a His tag at the C terminus. The expression construct was transfected into human umbilical vein endothelial cells (HUVECs) and HeLa and HEK293T cells for expression. An examination of the cell lysate by Western blot analysis did not show the expression of RdRp, which was due to lack of RdRp expression and not due to poor antibody reactivity. The gene was next cloned in pFastBac vector to examine the expression in Sf9 insect cells, using a baculovirus expression system. However, multiple trials revealed that RdRp was not expressed in insect cells as well (not shown). Hantavirus RdRp is a large protein of 250 kDa. To our knowledge, its structure is not known, although two functional domains have been predicted by *in silico* studies. Similar to other segmented negative-strand RNA viruses, the hantavirus RdRp initiates transcription by a unique cap-snatching mechanism [149-152, 189]. Based on *in slico* analysis, the regions around amino acids 1 to 250 and 751 to 1290 have been proposed to constitute the cap-snatching endonuclease domain and catalytic domain, respectively [54]. The regions from amino acids 251 to 750 and 1291 to 2153 have not been assigned any function (Fig. 8A) [52, 54]. However, the analysis by a domain prediction software that identifies conserved protein domains suggested the catalytic domain is slightly bigger and corresponds to the region from amino acids 562 to 1286. The upstream intervening sequence between the endonuclease domain and the catalytic domain constitutes the region from amino acids 238 to 562 (Fig. 8A). Since wild-type RdRp was not

expressed, we chose to individually express the predicted domains of RdRp in mammalian cells and examine their interaction with N protein. We generated constructs expressing the N-terminal endonuclease domain (pol1–250), intervening region of unknown function (pol251–752), catalytic domain (pol751–1290), and the C-terminal region of unknown function (pol1291–2153) (Fig. 8A). Due to ambiguity in the domain prediction, two additional constructs expressing the intervening region (pol238–562) and catalytic domain (pol562–1286) were also generated (Fig. 8A). All of these RdRp fragments contained a C-terminal His tag. HEK293T cells were cotransfected with plasmids expressing the RdRp fragment of interest along with C-terminally myc-tagged N protein. An examination of whole-cell lysates (WCL) by Western blot analysis showed that all RdRp fragments were expressed in HEK293T cells, except the pol1–250 fragment. In addition, the pol1291–2153 fragment was truncated from the N terminus (Fig. 8B). The Western blot analysis of whole-cell lysates using anti-myc antibody revealed the similar expression of N protein in cotransfected HEK293T cells, except for the cells coexpressing N protein along with the pol1–250 fragment. Based on these observations, it is clear that the N-terminal endonuclease domain does not accumulate in cells, and it also inhibited the accumulation of N protein in cotransfected cells. To determine whether the pol1–250 fragment specifically inhibited N protein expression, we cotransfected HEK293T cells with plasmids expressing the pol1–250 fragment along with another plasmid expressing either GFP or the luciferase reporter. Interestingly, we observed that the endonuclease domain nonspecifically inhibited the

expression of both reporters (not shown). An examination of whole-cell lysates (WCL) by Western blot analysis revealed that expression of the pol1–250 fragment also impacted the expression of endogenous proteins, such as GAPDH and tubulin (Fig. 8B). Recently, it has been suggested that the endonuclease domain of Andes virus RdRp cleaves the host cell transcripts and also its own mRNA and thereby regulates its own as well as host gene expression [70]. It is possible that endonuclease activity maintains the lower steady-state levels of RdRp in virus-infected cells, which is required for efficient virus replication. As mentioned later in this article, we expressed and purified the pol1–250 fragment in Escherichia coli. The purified fragment cleaved both capped and uncapped mRNAs nonspecifically *in vitro* (not shown). Moreover, overexpression of the pol1–250 fragment showed cytotoxicity in HeLa cells (Fig. 8C). Based on these observations, it is likely that endonuclease activity of the pol1–250 fragment suppressed its own as well as N protein expression in HEK293T cells (Fig. 8B).

To determine whether N protein interacts with any of the RdRp fragments, the cell lysates were immunoprecipitated with anti-myc antibody, and immunoprecipitated material was examined by Western blot analysis using anti-His tag antibody. It is evident from Fig. 8B that only the pol1291–2153 fragment copurified with N protein. Interestingly, the cleavage product of this fragment also copurified with N protein. Based on the size of the cleavage product, it is likely that the N protein binding domain is located at the C-terminal 400 amino acids of RdRp, although this needs further verification. To confirm these observations, cell lysates were incubated with Ni-nitrilotriacetic acid (NTA) beads, and the

eluted material from washed beads was examined by Western blot analysis using anti-myc tag antibody. This experiment further verified the interaction between the pol1291–2153 fragment and N protein (Fig. 8B). Since the pol1–250 fragment was not expressed in transfected cells, we expressed and purified both pol1–250 and pol1291–2153 fragments in E. coli using Ni-NTA beads. Cell lysates from HEK293T cells expressing C-terminally myc-tagged N protein were incubated with either the purified pol1–250 or pol1291–2153 fragment. The mixture was immunoprecipitated with anti-myc antibody, and immunoprecipitated material was examined by Western blot analysis using anti-His tag antibody. It is evident from Fig. 8D that unlike the pol1291–2153 fragment, the pol1–250 fragment did not bind to N protein. Also, the purified pol1291–2153 fragment from E. coli did not contain the truncation product, suggesting that truncation either specifically occurs in mammalian cells or the truncation product is unstable in E. coli or was lost during the purification process.

To reexamine the interaction between N protein and the C-terminal uncharacterized fragment of RdRp in hantavirus-infected cells, we infected Huh7 cells with SNV at a multiplicity of infection (MOI) of 2.0, followed by transfection with a plasmid expressing the C-terminally His-tagged pol1291–2153 fragment. Cell lysates were incubated with Ni-NTA beads. An examination of the eluted material from Ni-NTA beads using Western blot analysis confirmed the interaction between N protein and the pol1291–2153 fragment (Fig. 8D, top panel). To further confirm this interaction in virus-infected cells, the cell lysates were immunoprecipitated with anti-N protein antibody, and immunoprecipitated

material was examined by Western blot analysis using anti-His tag antibody. It is evident from Fig. 8E (bottom panel) that N protein interacts with the pol1291–2153 fragment in virus-infected cells.

To directly visualize the interaction between N protein and the pol1291–2153 fragment, we fused GFP and mCherry reporters at the N terminus of N protein and C terminus of the pol1291–2153 fragment, respectively. Similarly, mCherry was also fused at the C terminus of the pol238–562 fragment and used as a negative control. HeLa cells were cotransfected with plasmids expressing either the GFP-N fusion protein along with another plasmid expressing either the pol1291–2153 or pol238–562 fragment fused to mCherry. Cells were examined under a confocal microscope. As shown in Fig. 9A, both RdRp fragments and N protein showed the perinuclear punctate morphology. The green and red puncta representing N protein and the pol1291–2153 fragment strongly colocalized with each other (Fig. 9A). In comparison, we did not observe a noticeable colocalization between N protein and the pol238–562 fragment (Fig. 9A). These results are consistent with the specific binding of N protein to the pol1291–2153 fragment. To visualize the interaction between N protein and the pol1291–2153 fragment in virus-infected cells, we infected Vero E6 cells with SNV at an MOI of 2.0. Thirty-six hours postinfection, cells were transfected with a plasmid expressing the pol1291–2153 fragment fused to mCherry, as mentioned above. N protein was visualized in virus-infected cells by confocal microscopy using a fluorescein isothiocyanate (FITC)-conjugated antibody. As shown in Fig. 9B, N protein showed a rod-shaped perinuclear morphology, presumably representing

the virion RNPs. Again, a noticeable colocalization between the pol1291–2153 fragment and N protein was observed. The expression of RdRp fragments pol238–562 or pol1291–2153 does not seem to influence the N protein morphology in virus-infected cells. These results clearly demonstrate that N protein interacts with the C-terminal uncharacterized fragment of RdRp.

To identify the RdRp binding domain in the N protein, we used a panel of both the C- and N-terminal deletion mutants of the N protein (Fig. 10A). All of these mutants contained a C-terminal His tag. We also modified the pol1291–2153 expression construct (Fig. 2A) by replacing the C-terminal His tag with a FLAG tag. HEK293T cells were cotransfected with a plasmid expressing the FLAG-tagged pol1291–2153 fragment along with another plasmid expressing the N mutant of interest. An examination of whole-cell lysates (WCL) by Western blot analysis using anti-N protein antibody revealed that expression levels of few N mutants were weaker than those of other ones (Fig. 10B), although differential recognition of N protein mutants by anti-SNV N antibody cannot be ruled out. However, the levels of expression of the FLAG-tagged pol1291–2153 fragment in whole-cell lysates were relatively similar in all samples. Cell lysates were immunoprecipitated by anti-FLAG tag antibody, and immunoprecipitated material was examined by Western blot analysis using anti-N protein antibody. As shown in Fig. 10B, that deletion of up to 252 amino acids from the C terminus (N1–175 mutant) did not abrogate the interaction between N protein and pol1291–2153 fragment. In comparison, the deletion of just 50 amino acids from the N terminus of N protein abrogated the interaction. To further confirm these results, we

incubated the cell lysates with Ni-NTA beads, and the bound material eluted from washed beads was examined by Western blot analysis using anti-FLAG antibody. It is evident that unlike the N-terminal deletion mutants, all of the C-terminal deletion mutants bound to FLAG-tagged pol1291–2153 fragment, consistent with similar observations from the immunoprecipitation experiment. Taken together, these results suggest that the first 50 amino acids at the N terminus of N protein constitute the RdRp binding domain.

To determine whether an N-RdRp interaction plays a role in hantavirus replication, we transfected Huh7 cells with a plasmid expressing either the pol1291–2153 or pol751–1290 fragment, followed by infection with SNV at an MOI of 0.5 16 h posttransfection. Cells were transfected again 36 h postinfection to boost the expression of RdRp fragments. Virus replication was monitored over time by quantitative estimation of viral S-segment RNA using real-time PCR analysis, as previously reported [190]. It is evident that expression of the pol1291–2153 fragment significantly inhibited the SNV replication in cells (Fig. 11A). In comparison, the coexpression of the pol751–1290 fragment, which does not bind to N, had no impact upon virus replication. To determine whether the pol1291–2153 fragment selectively inhibits SNV replication, we examined the effect of this fragment upon the replication of vesicular stomatitis virus (VSV) (Fig. 11B) , another negative-strand RNA virus, in the cell culture model. HeLa cells grown in 24-well plates were transfected with a plasmid expressing the pol1291–2153 fragment, followed by infection with VSV at 10^3 PFU 16 h posttransfection. An examination of virus replication over time revealed that

coexpression of the pol1291–2153 fragment had no impact upon the replication of VSV, confirming the selectivity inhibition of SNV N by the pol1291–2153 fragment. Similar results were obtained with hepatitis C virus (HCV) (data not shown).

Due to low expression levels, hantavirus RdRp has never been shown by Western blot analysis in virus-infected cells. We speculate that overexpression of the pol1291–2153 fragment in virus-infected cells (Fig. 11A) outcompeted wild-type RdRp for binding to N protein, which resulted in the inhibition of wild-type RdRp function. This clearly demonstrates that the N-RdRp interaction plays a critical role in the function of RdRp, although the mechanism for such a role is still unclear. This is consistent with requirement of N protein expression for RdRp function in numerous Bunyaviridae reverse genetic systems[139, 191]. We propose that an N-RdRp interaction might play a role in cap snatching. Recently, multiple studies have identified the functional manganese-dependent endonuclease domain at the N terminus of the Bunyaviridae RdRp that shares a type II endonuclease α/β architecture similar to that of the N-terminal endonuclease domain of the influenza virus PA subunit[52, 67, 153, 163, 192, 193]. These studies have led to the proposition that the RdRp endonuclease domain functions in cap snatching and is highly conserved among the Arenaviridae, Orthomyxoviridae, and Bunyaviridae families. Interestingly, the purified endonuclease domain used in these studies [52, 163] nonspecifically cleaved the RNA irrespective of the 5′ cap, raising the question of how capped primers of the appropriate length and specificity are generated by such a

nonspecific cap-snatching endonuclease. The cap-binding activity of hantavirus RdRp has not been reported yet, again raising the question of how RdRp is loaded onto the mRNA 5′ cap during cap snatching. We previously reported that N protein binds to the mRNA 5′ cap. It is likely that simultaneous binding of N protein to both the RdRp and the mRNA 5′ cap may recruit RdRp at the mRNA 5′ cap for the specific cleavage of capped host cell mRNA to generate RNA primers of the appropriate length and specificity. It is possible that overexpressed pol1291–2153 fragment (Fig. 11A) competitively inhibited the binding of wild-type RdRp with N protein in virus-infected cells, which culminated in the inhibition of the cap-snatching process. It is equally possible that RdRp is recruited to the nucleocapsid templates by the N-RdRp interaction during transcription and replication of the viral genome. The strong colocalization of the overexpressed pol1291–2153 fragment with N protein in virus-infected cells suggests that the pol1291–2153 fragment might have competitively blocked the recruitment of wild-type RdRp on the nucleocapsid templates for transcription initiation.

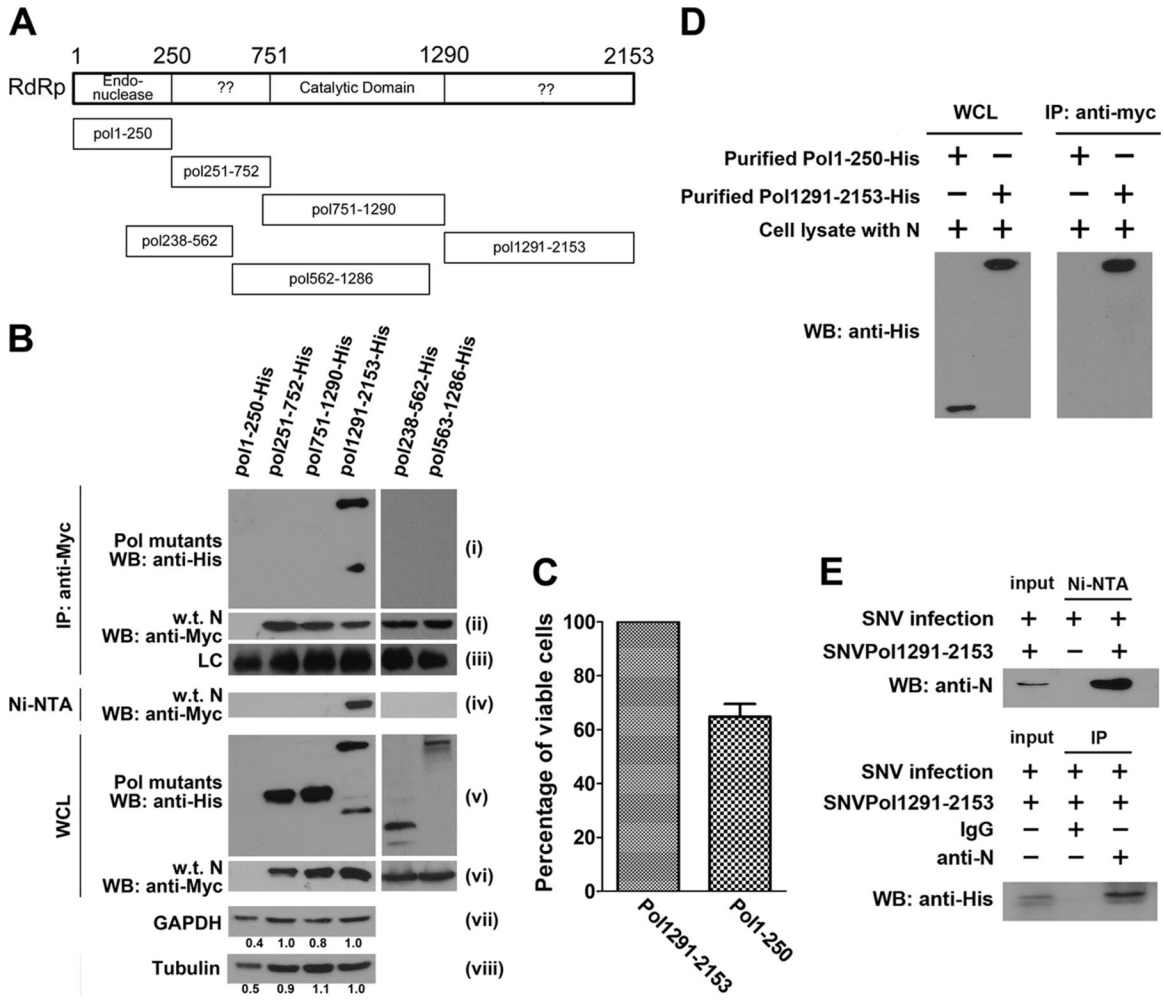

A
1
250
751
1290
2153
RdRp
Endo-nuclease
??
Catalytic Domain
??
pol1-250
pol251-752
pol751-1290
pol238-562
pol1291-2153
pol562-1286
B
pol1-250-His
pol251-752-His
pol751-1290-His
pol1291-2153-His
pol238-562-His
pol563-1286-His
IP: anti-Myc
Pol mutants WB: anti-His
(i)
w.t. N WB: anti-Myc
(ii)
LC
(iii)
Ni-NTA
w.t. N WB: anti-Myc
(iv)
WCL
Pol mutants WB: anti-His
(v)
w.t. N WB: anti-Myc
(vi)
GAPDH
(vii)
0.4 1.0 0.8 1.0
Tubulin
(viii)
0.5 0.9 1.1 1.0
C
Percentage of viable cells
100
80
60
40
20
0
Pol1291-2153
Pol1-250
D
WCL
IP: anti-myc
Purified Pol1-250-His
Purified Pol1291-2153-His
Cell lysate with N
WB: anti-His
E
input
Ni-NTA
SNV infection
SNVPol1291-2153
WB: anti-N
input
IP
SNV infection
SNVPol1291-2153
IgG
anti-N
WB: anti-His

Figure 8 Interaction of N with RdRp.

(A) Pictorial representation of RdRp mutants used in this study. A conserved domain prediction software from NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) suggested that the catalytic domain is located in the region from amino acids 562 to 1286 and the upstream intervening region of unknown function corresponds to the region from amino acids 238 to 562. (B) HEK293T cells grown on 60-mm-diameter dishes were cotransfected with a plasmid expressing myc-tagged wild-type (w.t.) N protein along with another plasmid expressing the His-tagged RdRp fragment of interest (pol1–250, pol251–752, pol751–1290, pol1291–2153, pol238–562, or pol562–1286). Cells were lysed 48 h posttransfection, the resulting cell lysates were immunoprecipitated (IP) with anti-myc tag antibody, and immunoprecipitated material was examined by Western blot (WB) analysis using either anti-His tag antibody (panel i) or anti-myc tag antibody (panel ii). The light chain of anti-myc antibody is shown in panel iii. Cell lysates were also incubated with Ni-NTA beads, and the eluted material from washed beads was examined by Western blot analysis using anti-myc tag antibody (panel iv). Equal volumes of whole-cell lysates (WCL) were examined by Western blot analysis using either anti-His tag antibody (panel v) or anti-myc tag antibody (panel vi) or anti-GAPDH antibody (panel vii) or antitubulin antibody (panel viii). The band intensity for β-actin and tubulin was quantified and normalized to the last band from the left; the intensity is indicated at the bottom. Note that the plasmids used for transfection are shown in Table 1. (C) HeLa cells in a 96-well plate at 70% confluence were transfected

with 2.5 μg of a plasmid expressing either the pol1–250 fragment or pol 238–562 fragment. Cell viability was measured 48 h posttransfection using the CellTox green cytotoxicity assay. The experiment was performed in duplicates. (D) Both pol1–250 and pol1291–2153 fragments were expressed in E. coli and purified using Ni-NTA beads. HEK293T cells were transfected with a plasmid expressing wild-type N protein. Cells were lysed 48 h posttransfection, and the resulting lysates were incubated with 2 μg of the purified RdRp fragment of interest at 4°C for 3 h. The lysates were immunoprecipitated with anti-myc tag antibody, and the immunoprecipitated material was analyzed by Western blotting using anti-His tag antibody to detect the RdRp fragments. (E) Huh7 cells in a 60-mm dish were infected with SNV at an MOI of 2. Twenty-four hours postinfection, cells were transfected with 2.5 μg of a plasmid expressing the Pol1291–2153 fragment. Cells were lysed, the resulting lysates were immunoprecipitated with polyclonal anti-N antibody, and the immunoprecipitated material was analyzed by Western blot analysis using anti-His antibody to detect the pol1291–2153 fragment (bottom). The cell lysates were also incubated with Ni-NTA beads, and the eluted material from washed beads was analyzed by Western blotting using anti-N antibody (top).

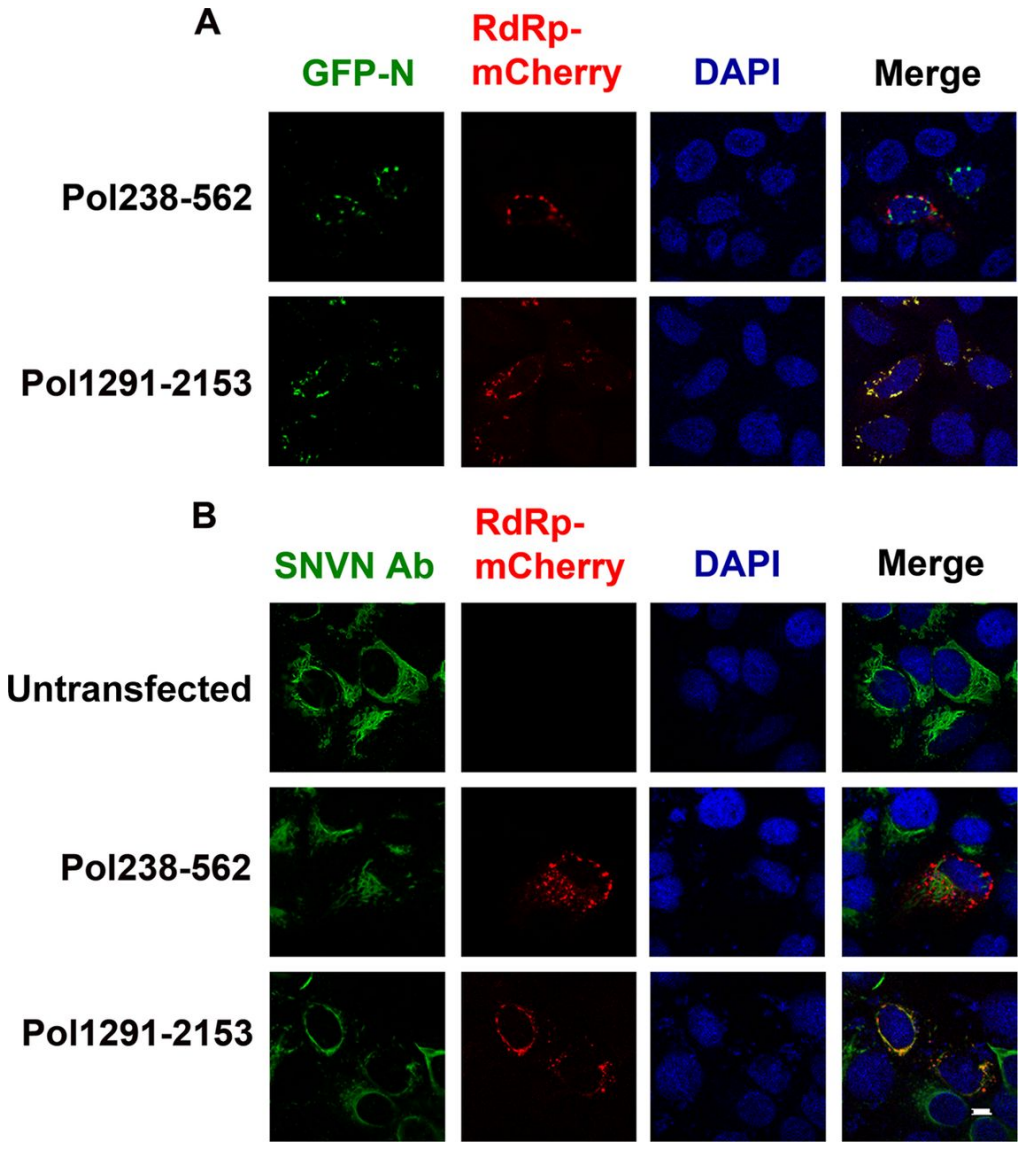

A
GFP-N
RdRp-mCherry
DAPI
Merge
Pol238-562
Pol1291-2153
B
SNVN Ab
RdRp-mCherry
DAPI
Merge
Untransfected
Pol238-562
Pol1291-2153

Figure 9 Confocal imaging of N and RdRp fragments.

(A) HeLa cells were grown on a coverslip up to 70% confluence in a 35-mm-diameter dish. Cells were cotransfected with a plasmid expressing GFP-N fusion protein along with another plasmid expressing either the pol238–562 or pol1293–2153 fragment fused with mCherry. Thirty-six hours posttransfection, cells were fixed with 3.7% paraformaldehyde (PFA) and visualized with a Nikon Eclipse C1si confocal microscope. Due to the large image size, all images in each row were stacked, and the same region was excised for presentation in this figure. DAPI, 4′,6-diamidino-2-phenylindole. (B) We next examined the colocalization of N protein with pol238–562 and pol1291–2153 fragments in virus-infected cells. Vero E6 cells were grown on a coverslip as mentioned above and infected with SNV at the MOI of 2. Thirty-six hours postinfection, cells were transfected with 2.5 μg of plasmid expressing either the pol238–562 or pol1293–2153 fragment fused to mCherry. Eighteen hours posttransfection, cells were fixed and examined under confocal microscope. N protein was visualized using anti-N primary antibody and a secondary antibody conjugated with FITC. In the control experiment, rat IgG was used instead of anti-N primary antibody, and the secondary antibody was conjugated with FITC, which did not generate any signal (not shown). This demonstrates the specificity of anti-N antibody in this assay. The bar in the right bottom panel is 10 μm.

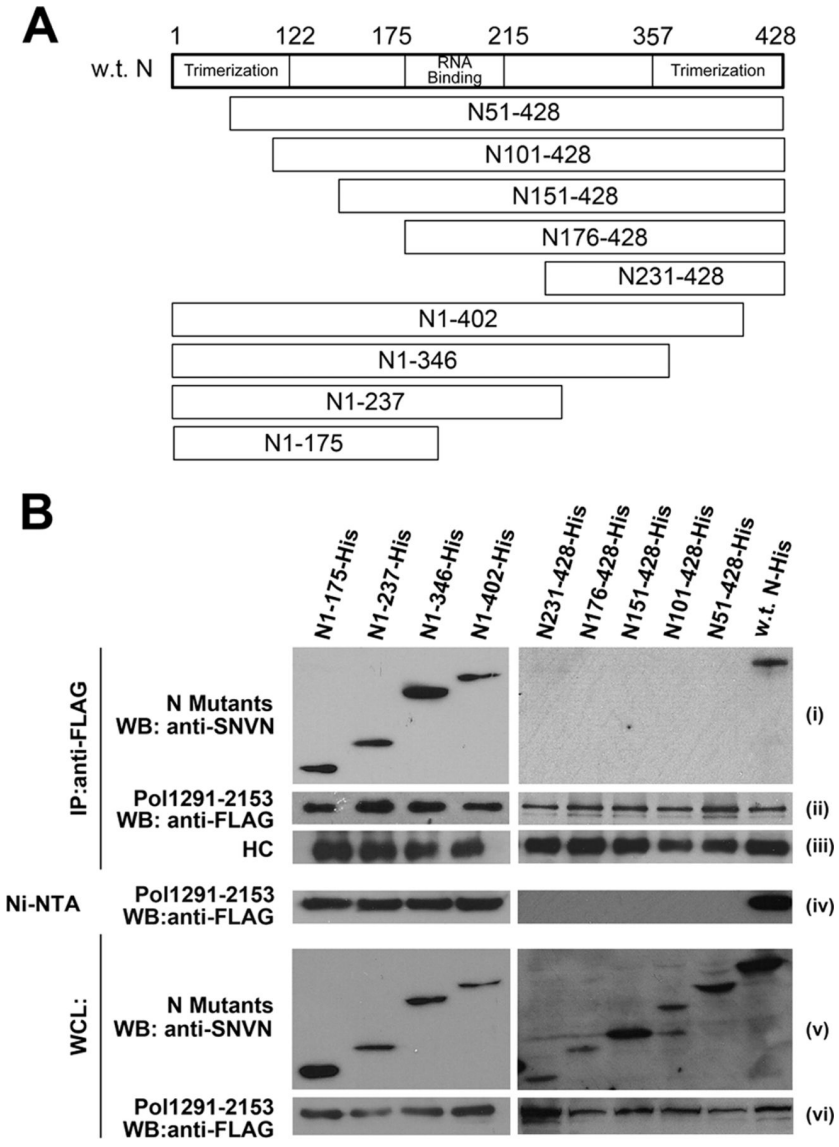

A
1
122
175
215
357
428
w.t. N
Trimerization
RNA Binding
Trimerization
N51-428
N101-428
N151-428
N176-428
N231-428
N1-402
N1-346
N1-237
N1-175
B
N1-175-His
N1-237-His
N1-346-His
N1-402-His
N231-428-His
N176-428-His
N151-428-His
N101-428-His
N51-428-His
w.t. N-His
IP:anti-FLAG
N Mutants
WB: anti-SNVN
(i)
Pol1291-2153
WB: anti-FLAG
(ii)
HC
(iii)
Ni-NTA
Pol1291-2153
WB:anti-FLAG
(iv)
WCL:
N Mutants
WB: anti-SNVN
(v)
Pol1291-2153
WB:anti-FLAG
(vi)

Figure 10 The RdRp binding domain is located at the N terminus of N. (A) Pictorial representation of N deletion mutants used in this work. The deleted regions are not shown. (B) HEK293T cells grown on 60-mm-diameter dishes were cotransfected with a plasmid (Table 1) expressing FLAG-tagged pol1291–2153 fragment along with another plasmid expressing the His-tagged wild type (w.t.) or N mutant (N51–428, N101–428, N151–428, N176–428 N231–428, N1–402, N1–346, N1–237, or N1–175). Forty-eight hours posttransfection, cells were lysed, the resulting cell lysates were immunoprecipitated (IP) with anti-FLAG antibody, and the immunoprecipitated material was examined by Western blotting (WB) using either anti-SNV N antibody to detect N mutants (panel i) or anti-FLAG antibody to detect pol1291–2153 fragment (panel ii). The heavy chain of anti-FLAG antibody is shown in panel iii. The cell lysates were also incubated with Ni-NTA slurry (Qiagen), and the eluted material from washed beads was examined by Western blotting using anti-FLAG antibody to detect the pol1291–2153 mutant (panel iv). Equal volumes of whole-cell lysates (WCL) were also examined by Western blot analysis using either anti-SNV N antibody (panel v) or anti-FLAG tag antibody (panel vi). Note that the plasmids used for transfection are shown in Table 1.

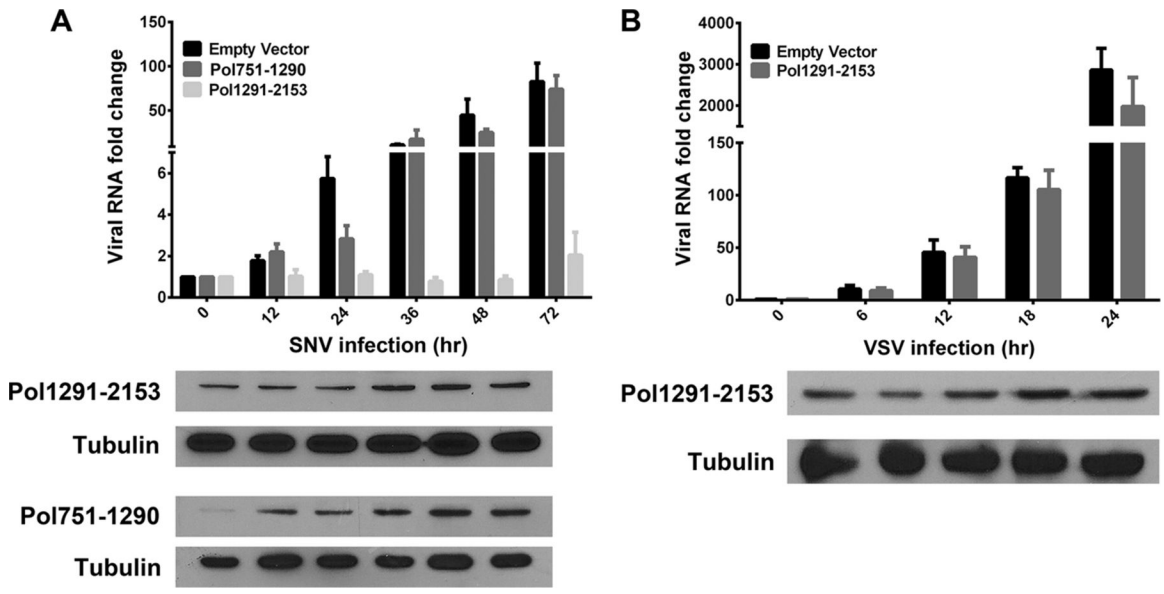

A
Viral RNA fold change
Empty Vector
Pol751-1290
Pol1291-2153
SNV infection (hr)
Pol1291-2153
Tubulin
Pol751-1290
Tubulin
B
Viral RNA fold change
Empty Vector
Pol1291-2153
VSV infection (hr)
Pol1291-2153
Tubulin

Figure 11 Overexpression of the pol1291–2153 mutant specifically inhibits hantavirus replication in cells.

(A) Huh7 cells were transfected with 2.5 μg of plasmid expressing either the pol1291–2153 or pol751–1290 mutant. Sixteen hours posttransfection, cells were infected with SNV at an MOI of 0.5. Thirty-six hours postinfection, cells were again transfected to boost the expression of RdRp fragments. Cells were harvested at 0, 12, 24, 36, 48, and 72 h postinfection. Total RNA was extracted from half of the cells collected at each time point with the RNeasy minikit and converted to cDNA using a random primer. S-segment RNA levels were quantified by real-time PCR using β-actin as an internal control, as previously reported[1, 186] .Fold changes in S-segment RNA levels related to the zero hour time point are shown. Fold changes calculated from three independent experiments were averaged and used to calculate the standard deviation, shown as error bars. The remaining half of the cells were lysed with 1× Laemmli sample buffer containing 2% SDS and analyzed by Western blotting using the monoclonal anti-His antibody to monitor the expression of RdRp mutants. (B) HeLa cells were transfected with 2.5 μg of either empty vector or a plasmid expressing the Pol1291–2153 fragment. Cells were infected with vesicular stomatitis virus 16 h posttransfection (103 PFU/well in a 24-well plate). Cells were harvested at 0, 6, 12, 18, and 24 h postinfection. Half of the cells were used for RNA extraction to quantify the VSV genomic RNA levels using real-time PCR. Fold changes in vRNA levels were calculated as mentioned in panel A. The remaining half of the cells were lysed for Western blot analysis to check the

expression of tubulin and the pol1291–2153 fragment (bottom), as described in panel A.

Plasmid	Sequence of primer		
	Forward	Reverse	Backbone
pN51-428-His	CATG**CCATGG**TGTCTGCATTGGAGACCAAACTCG	TGGTGGTG**CTCGAG**TTTAAGTGGTTCTTGGTTAGAGATTTCC	pTrix1.1
pN101-428-His	CATG**CCATGG**TCCTTGATGTAAATTCCATTGACT	TGGTGGTG**CTCGAG**TTTAAGTGGTTCTTGGTTAGAGATTTCC	pTrix1.1
pN151-428-His	CATG**CCATGG**AAAATAAGGGAACAAGAATCCGATT	TGGTGGTG**CTCGAG**TTTAAGTGGTTCTTGGTTAGAGATTTCC	pTrix1.1
pN176-428-His	CATG**CCATGG**GACATCTATATGTTTCTATGCCAAC	TGGTGGTG**CTCGAG**TTTAAGTGGTTCTTGGTTAGAGATTTCC	pTrix1.1
pN231-428-His	CATG**CCATGG**ATTGGATGGAAAGGATTGATGACT	TGGTGGTG**CTCGAG**TTTAAGTGGTTCTTGGTTAGAGATTTCC	pTrix1.1
pN1-175-His	CTAG**CCATGG**GCACCCTCAAAGAAGTGCAAG	TATAAT**CTCGAG**TGGCTTACGTATTCCATTAACT	pTrix1.1
pN1-237-His	CTAG**CCATGG**GCACCCTCAAAGAAGTGCAAG	TATAAT**CTCGAG**ATCATCCTTGAATCGGATTCTT	pTrix1.1
pN1-346-His	CTAG**CCATGG**GCACCCTCAAAGAAGTGCAAG	TATAAT**CTCGAG**AGATTTTGATGCCATTATGGTG	pTrix1.1
pN1-402-His	CTAG**CCATGG**GCACCCTCAAAGAAGTGCAAG	TATAAT**CTCGAG**ATCCATATCATCTCCAAGATGG	pTrix1.1
pPol1–250	ATAATAT**CCATGG**AGAAGTACCGCGAGATCC	ATAATAT**CTCGAG**CCAGTGCTTGCAGTACTGGATCAGG	pTrix1.1
pPol251–752	ATAATAT**CCATGG**TGACCGAGGATCACGATTTCGTGTTC	ATAATAT**CTCGAG**CGGGCCCACTCCACGGTC	pTrix1.1
pPol751–1290	ATAATAT**CCATGG**CCCGCAAGTTCGAGGCCAAG	ATAATAT**CTCGAG**GCCTTCACGCAGCGGCTCTG	pTrix1.1
pPol1291–2153	ATAATAT**CCATGG**CCTTCTACAGCTACAAGCACACCC	ATAATAT**CTCGAG**GTAGAAGCTGCTCACGGGATC	pTrix1.1
pPol238–562	ATATAT**CCATGG**CGCCCGAGATCACCAACCTGATCCAGT	ATATAT**CTCGAG**GATGCTCATGACCTTGCTGAAGCAC	pTrix1.1
pPol562–1286	ATATAT**CCATGG**CGATCGATCTGAACCGCCTGCTGGCCC	ATATAT**CTCGAG**ATCGGCCATGCCGATGCCGGCGGTG	pTrix1.1
pCDNA-FLAG	AATAATA**CTCGAG**GATTACAAGGAGTACGATGACAAGGAT TACAAGGATGACGATGACAAGGATTACAAGGAT	ATAATT**CTAGA**TTACTTGTCATCGTCATCCTTGTAATCCTTG TCATCGTCATCCTTGTAATCCTTGTATCGTC	pcDNA3.1(+)
pPol1291–2153-FLAG	ATAATAT**GCTAGC**GCCTTCTACAGCTACAAGCACACCC	ATAATAT**CTCGAG**GTAGAAGCTGCTCACGGGATC	pCDNA-FLAG
pTriEx-mCherry	TATAAT**CTCGAG**GTGAGCAAGGGCGAGGAGGATAACAT	TAATAT**GCATGC**TTACTTGTACAGCTCGTCCATGCCG	pTriEx1.1

Table 1 Constructs used in this study

To generate His-tagged N mutants, PCR product was generated from pSNVN using the appropriate primers listed above. The PCR product was cloned into pTriEX 1.1 between the NcoI and XhoI sites. To generate His-tagged RdRp mutants, the region of interest was PCR amplified using the appropriate primers listed above, and the PCR product was cloned into the pTriEX 1.1 backbone between the NcoI and XhoI sites. pCNDA-FLAG was modified from pCDNA3.1(+) by annealing the listed primers and inserting the resulting DNA into pCDNA3.1(+) between the XhoI and XbaI sites. pPol1291–2153-FLAG was generated from pPol1291–2153 using the appropriate primers listed above, and the PCR product was cloned into pCDNA-FLAG between NheI and XhoI. To generate pTriEx mCherry, the PCR product was generated from pmCherry using the appropriate primers listed above. The PCR product was cloned into the pTriEX 1.1 backbone between the XhoI and SphI sites. The pPol238–562-mCherry and pPol1291–2153-mCherry constructs were generated by excising the open reading frame (ORF) from pPol238–562 and pPol1291–2153 using the NcoI and XhoI

restriction enzymes. The excised ORF was cloned into pTriEx-mCherry between the same restriction sites.

Chapter IV

Conclusions and Discussion

With the negative strand RNA viruses such as Orthomyxoviridae (e.g., influenza viruses and Thogoto viruses), Bunyaviridae (e.g., La Crosse virus, Hantavirus, Rift Valley fever virus, and Crimean-Congo hemorrhagic fever viruse) and Arenaviridae (e.g., Lassa virus) family members, viral mRNA synthesis is initiated by a cap-snatching mechanism, which uses host capped mRNA as primers for mRNA synthesis [176, 177]. In hantavirus, transcription initiates through a “prime-and-realign” mechanism, in which a guanine residue from a cap donor undergo base-paring with a cysteine residues at the 3’ terminus of a vRNA segment [37, 69]. The initial alignment is followed by cleavage of cap donor after annealing site (G) and subsequent synthesis of nascent viral mRNA. However, not all guanine-containing capped-oligos are used as cap-donor equally. Sequencing studies showed non-viral leading sequence of bunyavirus mRNA is most ranging from 10-20 nucleotides long [157, 158]. Thus, it remains a mystery of how cap-donors are selected and processed by hantavirus. Our studies on hantavirus cap-snatching using test capped transcripts as cap-donor has revealed several characteristics of a preferred cap donor for the virus to prime its transcription. First, the cap donor is preferentially used by virus when the guanine residues that would anneal to the viral RNA is located around 14 nucleotides downstream of the 5’ cap. Second, there is a positive correlation between the cap-snatching efficiency and sequence complementarity of the nucleotides on

the capped oligo and the 3’ terminus of vRNA. These observations suggests that there is a specific selection of cap donors by hantavirus, which is strongly contributed by the annealing strength of vRNA and cap donor mRNAs.

The hantavirus replication cycle is confined to the cytoplasm. Therefore, the vRNPs have to effectively compete with the host mRNA processing machinery including those involve in translation and mRNA decay in order to acquire sufficient capped mRNA to be used as primers.

Our study observed that virus preferentially snatch caps from a PTC-containing mRNA. Moreover, we found the mRNA engaged in translation may not be a strong cap donor even if it contains one or more complementary nucleotides to the 3′ terminus of the viral RNA template at the appropriate position. We observed hantavirus has extremely poor cap-snatching efficiency on its own mRNA, which possess highest complementarity to the vRNA; unless it contains a PTC. These findings suggest that hantaviruses prefers to use mRNAs that are not engaged in translation as primers for transcription.

P-bodies are the intracellular loci where non-translating mRNAs are stored and degraded. Hantavirus N proteins actively protect the host mRNA caps from degradation, possibly through competing with the decapping machinery [37, 194]. The N protein has been shown to localize with the P-body, indicating a possible role of P-body in hantavirus life cycle.

Our study showed that when P-body is disrupted by knocking down of its structural components, the steady state level of a non-translating mRNAs is elevated, resulting a larger cap-donor pool in the cytoplasm. However, the virus does not snatch caps better from these mRNAs. P-bodies might be a preferred, but not the only site for cap-snatching. A possible explanation is that in the P-body, the local concentration of ribosome free capped RNA is much higher than that in the cytoplasm, thus it would be easier for the N or vRNP to recognize and align to the potential cap-donor mRNA. When P-body is disassembled, more RNAs are available as cap-donors, but the total concentration of ribosome-free capped mRNAs does not increase significantly, because the foci where the ribosome free mRNAs accumulate, is lost. Further experimentation is required to demonstrate the exact role of cellular P bodies in the cap-snatching process.

Similar characteristics of cap donors are also observed in influenza virus cap-snatching. First, influenza virus shows a clear preference for cap-donors harboring a 3' CGA sequence, and the optimal non-viral primer length appears to be 10-11 nucleotides [63]. Second, while base-pairing is a preferred determinant for cap donor selection, the cap-snatching efficiency do not further increase when base-paring is greater than 3 [63]. (Table 2) However, the cellular loci of cap-snatching for influenza virus is different. Transcription of influenza virus occurs within the nucleus, thus the cap donors are exclusively pre-mRNAs [155].

	Hantavirus	Influenza virus
Preferred snatching site	G	AG
Complementarity correlating to cap-snatching efficiency	9 or over	Up to 3
Source of cap donor	Cellular mRNAs	Pre-mRNAs
Potential competing host machinery	Translation and RNA degradation	Transcription

Table 2. Comparison of requirements for a preferred cap-snatching primer between hantavirus and influenza virus

Another mystery in hantavirus cap-snatching mechanism is that how vRNP selects and processes the cap donor RNA in a highly selective fashion. It is known that the capped mRNA are cleaved at the guanine residues 10-20 nucleotides from the 5' cap with 14th guanine the most preferred cleavage site. In contrast, the endonuclease domain of the bunyaviridae RdRp has been demonstrated to be sequence non-specific [52]. Thus, we speculated that the RdRp within the vRNP must be restrained to the 5' of cap donor RNA possibly the cap to achieve selectivity on the position of the guanine. However, cap-binding ability has not been described with the bunyavirus RdRp [52]. Since the hantavirus N protein is able to the bind cap, we proposed that N might load the RdRp to the mRNA and restrain it at the vicinity to the 5' cap. Our study revealed that N is able to bind to the C-terminal portion of the RdRp, and the binding is independent of RNA, as an N mutant lacking RNA binding domain is also able to bind RdRp. In addition, we showed that the binding between RdRp C-terminal fragment and N protein is specifically important to hantavirus, as overexpression of the RdRp C-terminal fragment significantly reduced the level of hantavirus RNA produced, but not other negative strand RNA viruses. A possible explanation is that the wild type RdRp is expressed in the viral infected cells at negligible level and would be outcompeted by the overexpressed C-terminal fragment for N protein binding. However, it is not possible to distinguish whether the dominant negative effect is at the level of transcription or replication of the virus.

Studies in other virus systems also supported our speculation that RdRp interacts with cap, possibly bridged by nucleocapsid protein, might contributes the specificity of the hantavirus RdRp endonuclease cleavage during cap-snatching. It is thought that the cap-binding RdRp subunit of influenza virus, PB2, may play an important role in contributing the specific cleavage of mRNA substrate [64, 66, 68]. Both activity and sequence specificity of the endonuclease is significantly higher in the viral RNA than PA itself. This ssuggests a critical role of cap-binding mediated distance measurement from the 5' end of the RNA that contributes to the specificity of the influenza polymerase endonuclease.[68]

However, in case of hantavirus we have not yet demonstrated whether N-RdRp binding contributes to the specific cleavage of the capped donor in hantavirus as the full-length hantavirus RdRp cannot be expressed at a detectable level for purification. Therefore, further *in vitro* study using purified full-length RdRp is required to demonstrate the interaction between N and RdRp and its role in the specific cleavage of mRNA by the RdRp endonuclease.

Taken together, we propose a model of a possible mechanism by which hantavirus achieves cap donor selection and specific cleavage (Fig. 1) This proposed model suggests a novel mechanism of cap-snatching in bunyaviridae family viruses. More importantly, our study revealed novel anti-targets against hantavirus, and will facilitate the development of treatment strategies for hantavirus-caused diseases.

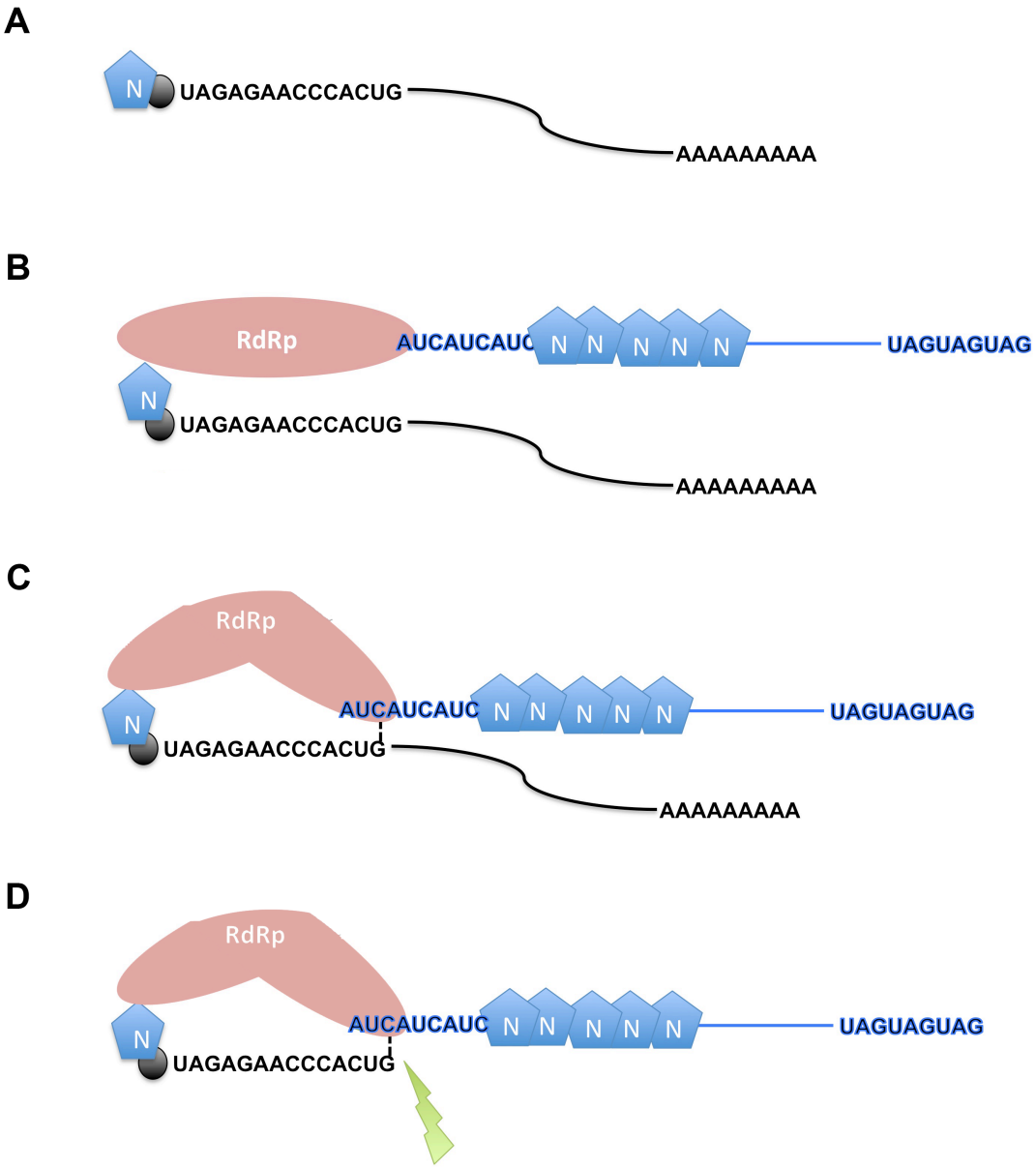

A
N
UAGAGAACCCACUG
AAAAAAAAA
B
RdRp
AUCAUCAUC
N N N N N
UAGUAGUAG
N
UAGAGAACCCACUG
AAAAAAAAA
C
RdRp
AUCAUCAUC
N N N N N
UAGUAGUAG
N
UAGAGAACCCACUG
AAAAAAAAA
D
RdRp
AUCAUCAUC
N N N N N
UAGUAGUAG
N
UAGAGAACCCACUG

Figure 12 A proposed model for hantavirus cap-snatching mechanism

The specific cap-snatching is proposed to go through the following steps: (A) N binds to the 5’ cap of the cap donor RNA and protects it from degradation. (B) RdRp associated with viral RNP is loaded to the cap donor through N-RdRp interaction. (C) vRNP scans the cap donor RNA in a 5’ to 3’ fashion for nucleotides complimentary to 3’ terminus (predominantly G residue) and followed by base-paring annealing of vRNA and cap donor. The range of distance of guanine residue is limited due to the size and flexibility of the RdRp protein. (D) vRNP cleaves cap-donor after the annealing sequence. Only cap donor is cleaved as vRNA is covered by N proteins.

References List

1. Vaheri, A., Mills, J. N., Spiropoulou, C. F. & Hjelle, B., *Oxford Textbook of Zoonoses: Biology, Clinical Practice and Public Health Control*, ed. S.R. almer, Lord Soulsby, Torgerson, P. R. & Brown, D. W. G. 2011: Oxford Univ. Press.

2. Hjelle, B. and F. Torres-Perez, *Hantaviruses in the americas and their role as emerging pathogens.* Viruses, 2010. **2**(12): p. 2559-86.

3. Jonsson, C.B., L.T. Figueiredo, and O. Vapalahti, *A global perspective on hantavirus ecology, epidemiology, and disease.* Clin Microbiol Rev, 2010. **23**(2): p. 412-41.

4. Martinez, V.P., et al., *Person-to-person transmission of Andes virus.* Emerg Infect Dis, 2005. **11**(12): p. 1848-53.

5. Sinisalo, M., et al., *Headache and low platelets in a patient with acute leukemia.* J Clin Virol, 2010. **48**(3): p. 159-61.

6. Hooper, J.W., et al., *A lethal disease model for hantavirus pulmonary syndrome.* Virology, 2001. **289**(1): p. 6-14.

7. Vapalahti, O., et al., *Hantavirus infections in Europe.* Lancet Infect Dis, 2003. **3**(10): p. 653-61.

8. Duchin, J.S., et al., *Hantavirus pulmonary syndrome: a clinical description of 17 patients with a newly recognized disease. The Hantavirus Study Group.* N Engl J Med, 1994. **330**(14): p. 949-55.

9. Simpson, S.Q., et al., *Hantavirus pulmonary syndrome.* Infect Dis Clin North Am, 2010. **24**(1): p. 159-73.

10. Mackow, E.R. and I.N. Gavrilovskaya, *Hantavirus regulation of endothelial cell functions.* Thromb Haemost, 2009. **102**(6): p. 1030-41.

11. Kanerva, M., J. Mustonen, and A. Vaheri, *Pathogenesis of puumala and other hantavirus infections.* Rev Med Virol, 1998. **8**(2): p. 67-86.

12. Zaki, S.R., et al., *Hantavirus pulmonary syndrome. Pathogenesis of an emerging infectious disease.* Am J Pathol, 1995. **146**(3): p. 552-79.

13. Kanerva, M., et al., *Pulmonary involvement in nephropathia epidemica: radiological findings and their clinical correlations.* Clin Nephrol, 1996. **46**(6): p. 369-78.

14. Shrivastava-Ranjan, P., P.E. Rollin, and C.F. Spiropoulou, *Andes virus disrupts the endothelial cell barrier by induction of vascular endothelial growth factor and downregulation of VE-cadherin.* J Virol, 2010. **84**(21): p. 11227-34.

15. Gavrilovskaya, I., et al., *Elevated VEGF Levels in Pulmonary Edema Fluid and PBMCs from Patients with Acute Hantavirus Pulmonary Syndrome.* Adv Virol, 2012. **2012**: p. 674360.

16. Gavrilovskaya, I.N., E.E. Gorbunova, and E.R. Mackow, *Pathogenic hantaviruses direct the adherence of quiescent platelets to infected endothelial cells.* J Virol, 2010. **84**(9): p. 4832-9.

17. Krautkramer, E., et al., *Pathogenic old world hantaviruses infect renal glomerular and tubular cells and induce disassembling of cell-to-cell contacts.* J Virol, 2011. **85**(19): p. 9811-23.

18. Schmaljohn, C.S., et al., *Antigenic and genetic properties of viruses linked to hemorrhagic fever with renal syndrome.* Science, 1985. **227**(4690): p. 1041-4.

19. Schmaljohn, C.N., ST. , *Fields Virology*, ed. D.H. Knipe, PM. 2007: Lippincott Williams & Wilkins. 1741-1790.

20. Spiropoulou, C., *Bunyaviridae. Molecular and Cellular Biology*. 2011: Caister Academic Press.

21. Hussein, I.T., et al., *Recent advances in hantavirus molecular biology and disease.* Adv Appl Microbiol, 2011. **74**: p. 35-75.

22. Kaukinen, P., A. Vaheri, and A. Plyusnin, *Hantavirus nucleocapsid protein: a multifunctional molecule with both housekeeping and ambassadorial duties.* Arch Virol, 2005. **150**(9): p. 1693-713.

23. Xu, X., et al., *The RNA binding domain of the hantaan virus N protein maps to a central, conserved region.* J Virol, 2002. **76**(7): p. 3301-8.

24. Li, X.D., et al., *Hantavirus nucleocapsid protein interacts with the Fas-mediated apoptosis enhancer Daxx.* J Gen Virol, 2002. **83**(Pt 4): p. 759-66.

25. Kaukinen, P., A. Vaheri, and A. Plyusnin, *Non-covalent interaction between nucleocapsid protein of Tula hantavirus and small ubiquitin-related modifier-1, SUMO-1.* Virus Res, 2003. **92**(1): p. 37-45.

26. Lee, B.H., et al., *Association of the nucleocapsid protein of the Seoul and Hantaan hantaviruses with small ubiquitin-like modifier-1-related molecules.* Virus Res, 2003. **98**(1): p. 83-91.

27. Maeda, A., et al., *The intracellular association of the nucleocapsid protein (NP) of hantaan virus (HTNV) with small ubiquitin-like modifier-1 (SUMO-1) conjugating enzyme 9 (Ubc9).* Virology, 2003. **305**(2): p. 288-97.

28. Gupta, S., et al., *Hantavirus-infection confers resistance to cytotoxic lymphocyte-mediated apoptosis.* PLoS Pathog, 2013. **9**(3): p. e1003272.

29. Taylor, S.L., R.L. Krempel, and C.S. Schmaljohn, *Inhibition of TNF-alpha-induced activation of NF-kappaB by hantavirus nucleocapsid proteins.* Ann N Y Acad Sci, 2009. **1171 Suppl 1**: p. E86-93.

30. Cimica, V., et al., *An innate immunity-regulating virulence determinant is uniquely encoded by the Andes virus nucleocapsid protein.* MBio, 2014. **5**(1).

31. Mir, M.A. and A.T. Panganiban, *A protein that replaces the entire cellular eIF4F complex.* EMBO J, 2008. **27**(23): p. 3129-39.

32. Mir, M.A., et al., *Hantavirus nucleocapsid protein has distinct m7G cap- and RNA-binding sites.* J Biol Chem, 2010. **285**(15): p. 11357-68.

33. Haque, A. and M.A. Mir, *Interaction of hantavirus nucleocapsid protein with ribosomal protein S19.* J Virol, 2010. **84**(23): p. 12450-3.

34. Mir, M.A. and A.T. Panganiban, *The triplet repeats of the Sin Nombre hantavirus 5' untranslated region are sufficient in cis for nucleocapsid-mediated translation initiation.* J Virol, 2010. **84**(17): p. 8937-44.

35. Skorko, R., D.F. Summers, and J.M. Galarza, *Influenza A virus in vitro transcription: roles of NS1 and NP proteins in regulating RNA synthesis.* Virology, 1991. **180**(2): p. 668-77.

36. Mir, M.A. and A.T. Panganiban, *Characterization of the RNA chaperone activity of hantavirus nucleocapsid protein.* J Virol, 2006. **80**(13): p. 6276-85.

37. Mir, M.A., et al., *Storage of cellular 5' mRNA caps in P bodies for viral cap-snatching.* Proc Natl Acad Sci U S A, 2008. **105**(49): p. 19294-9.

38. Wang, H., et al., *Interaction between hantaviral nucleocapsid protein and the cytoplasmic tail of surface glycoprotein Gn.* Virus Res, 2010. **151**(2): p. 205-12.

39. Ganaie, S.S. and M.A. Mir, *The role of viral genomic RNA and nucleocapsid protein in the autophagic clearance of hantavirus glycoprotein Gn.* Virus Res, 2014. **187**: p. 72-6.

40. Hussein, I.T., et al., *Autophagic clearance of Sin Nombre hantavirus glycoprotein Gn promotes virus replication in cells.* J Virol, 2012. **86**(14): p. 7520-9.

41. Hung, T., et al., *Morphology and morphogenesis of viruses of hemorrhagic fever with renal syndrome (HFRS). I. Some peculiar aspects of the morphogenesis of various strains of HFRS virus.* Intervirology, 1985. **23**(2): p. 97-108.

42. Martin, M.L., et al., *Distinction between Bunyaviridae genera by surface structure and comparison with Hantaan virus using negative stain electron microscopy.* Arch Virol, 1985. **86**(1-2): p. 17-28.

43. Goldsmith, C.S., et al., *Ultrastructural characteristics of Sin Nombre virus, causative agent of hantavirus pulmonary syndrome.* Arch Virol, 1995. **140**(12): p. 2107-22.

44. Huiskonen, J.T., et al., *Electron cryotomography of Tula hantavirus suggests a unique assembly paradigm for enveloped viruses.* J Virol, 2010. **84**(10): p. 4889-97.

45. Geimonen, E., et al., *Hantavirus pulmonary syndrome-associated hantaviruses contain conserved and functional ITAM signaling elements.* J Virol, 2003. **77**(2): p. 1638-43.

46. Tischler, N.D., et al., *Hantavirus Gc glycoprotein: evidence for a class II fusion protein.* J Gen Virol, 2005. **86**(Pt 11): p. 2937-47.

47. Estrada, D.F., et al., *The Hantavirus Glycoprotein G1 Tail Contains Dual CCHC-type Classical Zinc Fingers.* J Biol Chem, 2009. **284**(13): p. 8654-60.

48. Domingo, E.H., J.J., *Mutation rates and rapid evolution of RNA viruses*. 1994: New York: Raven Press.

49. Plyusnin, A., O. Vapalahti, and A. Vaheri, *Hantaviruses: genome structure, expression and evolution.* J Gen Virol, 1996. **77 (Pt 11)**: p. 2677-87.

50. Jonsson, C.B. and C.S. Schmaljohn, *Replication of hantaviruses.* Curr Top Microbiol Immunol, 2001. **256**: p. 15-32.

51. Jin, H. and R.M. Elliott, *Characterization of Bunyamwera virus S RNA that is transcribed and replicated by the L protein expressed from recombinant vaccinia virus.* J Virol, 1993. **67**(3): p. 1396-404.

52. Reguera, J., F. Weber, and S. Cusack, *Bunyaviridae RNA polymerases (L-protein) have an N-terminal, influenza-like endonuclease domain, essential for viral cap-dependent transcription.* PLoS Pathog, 2010. **6**(9): p. e1001101.

53. Kukkonen, S.K., A. Vaheri, and A. Plyusnin, *Completion of the Tula hantavirus genome sequence: properties of the L segment and heterogeneity found in the 3' termini of S and L genome RNAs.* J Gen Virol, 1998. **79 (Pt 11)**: p. 2615-22.

54. Kukkonen, S.K., A. Vaheri, and A. Plyusnin, *L protein, the RNA-dependent RNA polymerase of hantaviruses.* Arch Virol, 2005. **150**(3): p. 533-56.

55. Butcher, S.J., et al., *A mechanism for initiating RNA-dependent RNA polymerization.* Nature, 2001. **410**(6825): p. 235-40.

56. Jin, H. and R.M. Elliott, *Mutagenesis of the L protein encoded by Bunyamwera virus and production of monospecific antibodies.* J Gen Virol, 1992. **73 (Pt 9)**: p. 2235-44.

57. Sankar, S. and A.G. Porter, *Point mutations which drastically affect the polymerization activity of encephalomyocarditis virus RNA-dependent RNA polymerase correspond to the active site of Escherichia coli DNA polymerase I.* J Biol Chem, 1992. **267**(14): p. 10168-76.

58. O'Reilly, E.K. and C.C. Kao, *Analysis of RNA-dependent RNA polymerase structure and function as guided by known polymerase structures and computer predictions of secondary structure.* Virology, 1998. **252**(2): p. 287-303.

59. Schmaljohn, C.S., et al., *Characterization of Hantaan virions, the prototype virus of hemorrhagic fever with renal syndrome.* J Infect Dis, 1983. **148**(6): p. 1005-12.

60. Kukkonen, S.K., A. Vaheri, and A. Plyusnin, *Tula hantavirus L protein is a 250 kDa perinuclear membrane-associated protein.* J Gen Virol, 2004. **85**(Pt 5): p. 1181-9.

61. Jaaskelainen, K.M., et al., *Tula and Puumala hantavirus NSs ORFs are functional and the products inhibit activation of the interferon-beta promoter.* J Med Virol, 2007. **79**(10): p. 1527-36.

62. Bouloy, M., S.J. Plotch, and R.M. Krug, *Globin mRNAs are primers for the transcription of influenza viral RNA in vitro.* Proc Natl Acad Sci U S A, 1978. **75**(10): p. 4886-90.

63. Geerts-Dimitriadou, C., et al., *Base-pairing promotes leader selection to prime in vitro influenza genome transcription.* Virology, 2011. **409**(1): p. 17-26.

64. Li, M.L., P. Rao, and R.M. Krug, *The active sites of the influenza cap-dependent endonuclease are on different polymerase subunits.* EMBO J, 2001. **20**(8): p. 2078-86.

65. Fechter, P., et al., *Two aromatic residues in the PB2 subunit of influenza A RNA polymerase are crucial for cap binding.* J Biol Chem, 2003. **278**(22): p. 20381-8.

66. Guilligay, D., et al., *The structural basis for cap binding by influenza virus polymerase subunit PB2.* Nat Struct Mol Biol, 2008. **15**(5): p. 500-6.

67. Dias, A., et al., *The cap-snatching endonuclease of influenza virus polymerase resides in the PA subunit.* Nature, 2009. **458**(7240): p. 914-8.

68. Datta, K., et al., *Characterization of PA-N terminal domain of Influenza A polymerase reveals sequence specific RNA cleavage.* Nucleic Acids Res, 2013. **41**(17): p. 8289-99.

69. Garcin, D., et al., *The 5' ends of Hantaan virus (Bunyaviridae) RNAs suggest a prime-and-realign mechanism for the initiation of RNA synthesis.* J Virol, 1995. **69**(9): p. 5754-62.

70. Heinemann, P., J. Schmidt-Chanasit, and S. Gunther, *The N terminus of Andes virus L protein suppresses mRNA and protein expression in mammalian cells.* J Virol, 2013. **87**(12): p. 6975-85.

71. Ramanathan, H.N., et al., *Dynein-dependent transport of the hantaan virus nucleocapsid protein to the endoplasmic reticulum-Golgi intermediate compartment.* J Virol, 2007. **81**(16): p. 8634-47.

72. Eulalio, A., et al., *P-body formation is a consequence, not the cause, of RNA-mediated gene silencing.* Mol Cell Biol, 2007. **27**(11): p. 3970-81.

73. Sheth, U. and R. Parker, *Decapping and decay of messenger RNA occur in cytoplasmic processing bodies.* Science, 2003. **300**(5620): p. 805-8.

74. Cougot, N., S. Babajko, and B. Seraphin, *Cytoplasmic foci are sites of mRNA decay in human cells.* J Cell Biol, 2004. **165**(1): p. 31-40.

75. Andrei, M.A., et al., *A role for eIF4E and eIF4E-transporter in targeting mRNPs to mammalian processing bodies.* RNA, 2005. **11**(5): p. 717-27.

76. Muhlrad, D. and R. Parker, *The yeast EDC1 mRNA undergoes deadenylation-independent decapping stimulated by Not2p, Not4p, and Not5p.* EMBO J, 2005. **24**(5): p. 1033-45.

77. Parker, R. and U. Sheth, *P bodies and the control of mRNA translation and degradation.* Mol Cell, 2007. **25**(5): p. 635-46.

78. Franks, T.M. and J. Lykke-Andersen, *The control of mRNA decapping and P-body formation.* Mol Cell, 2008. **32**(5): p. 605-15.

79. Teixeira, D., et al., *Processing bodies require RNA for assembly and contain nontranslating mRNAs.* RNA, 2005. **11**(4): p. 371-82.

80. Liu, J., et al., *MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies.* Nat Cell Biol, 2005. **7**(7): p. 719-23.

81. Koritzinsky, M., et al., *Gene expression during acute and prolonged hypoxia is regulated by distinct mechanisms of translational control.* EMBO J, 2006. **25**(5): p. 1114-25.

82. Kedersha, N., et al., *Stress granules and processing bodies are dynamically linked sites of mRNP remodeling.* J Cell Biol, 2005. **169**(6): p. 871-84.

83. Brengues, M., D. Teixeira, and R. Parker, *Movement of eukaryotic mRNAs between polysomes and cytoplasmic processing bodies.* Science, 2005. **310**(5747): p. 486-9.

84. Ferraiuolo, M.A., et al., *A role for the eIF4E-binding protein 4E-T in P-body formation and mRNA decay.* J Cell Biol, 2005. **170**(6): p. 913-24.

85. Barbee, S.A., et al., *Staufen- and FMRP-containing neuronal RNPs are structurally and functionally related to somatic P bodies.* Neuron, 2006. **52**(6): p. 997-1009.

86. Coller, J. and R. Parker, *General translational repression by activators of mRNA decapping.* Cell, 2005. **122**(6): p. 875-86.

87. Decker, C.J., D. Teixeira, and R. Parker, *Edc3p and a glutamine/asparagine-rich domain of Lsm4p function in processing body assembly in Saccharomyces cerevisiae.* J Cell Biol, 2007. **179**(3): p. 437-49.

88. Franks, T.M. and J. Lykke-Andersen, *TTP and BRF proteins nucleate processing body formation to silence mRNAs with AU-rich elements.* Genes Dev, 2007. **21**(6): p. 719-35.

89. Bonnerot, C., R. Boeck, and B. Lapeyre, *The two proteins Pat1p (Mrt1p) and Spb8p interact in vivo, are required for mRNA decay, and are functionally linked to Pab1p.* Mol Cell Biol, 2000. **20**(16): p. 5939-46.

90. Bouveret, E., et al., *A Sm-like protein complex that participates in mRNA degradation.* EMBO J, 2000. **19**(7): p. 1661-71.

91. Coller, J.M., et al., *The DEAD box helicase, Dhh1p, functions in mRNA decapping and interacts with both the decapping and deadenylase complexes.* RNA, 2001. **7**(12): p. 1717-27.

92. Fischer, N. and K. Weis, *The DEAD box protein Dhh1 stimulates the decapping enzyme Dcp1.* EMBO J, 2002. **21**(11): p. 2788-97.

93. Tharun, S., et al., *Yeast Sm-like proteins function in mRNA decapping and decay.* Nature, 2000. **404**(6777): p. 515-8.

94. Tharun, S. and R. Parker, *Targeting an mRNA for decapping: displacement of translation factors and association of the Lsm1p-7p complex on deadenylated yeast mRNAs.* Mol Cell, 2001. **8**(5): p. 1075-83.

95. Reijns, M.A., et al., *A role for Q/N-rich aggregation-prone regions in P-body localization.* J Cell Sci, 2008. **121**(Pt 15): p. 2463-72.

96. Tritschler, F., et al., *A divergent Sm fold in EDC3 proteins mediates DCP1 binding and P-body targeting.* Mol Cell Biol, 2007. **27**(24): p. 8600-11.

97. Jakymiw, A., et al., *Disruption of GW bodies impairs mammalian RNA interference.* Nat Cell Biol, 2005. **7**(12): p. 1267-74.

98. Liu, J., et al., *A role for the P-body component GW182 in microRNA function.* Nat Cell Biol, 2005. **7**(12): p. 1261-6.

99. Yu, J.H., et al., *Ge-1 is a central component of the mammalian cytoplasmic mRNA processing body.* RNA, 2005. **11**(12): p. 1795-802.

100. Beelman, C.A. and R. Parker, *Differential effects of translational inhibition in cis and in trans on the decay of the unstable yeast MFA2 mRNA.* J Biol Chem, 1994. **269**(13): p. 9687-92.

101. Bhattacharyya, S.N., et al., *Relief of microRNA-mediated translational repression in human cells subjected to stress.* Cell, 2006. **125**(6): p. 1111-24.

102. Yedavalli, V.S., et al., *Requirement of DDX3 DEAD box RNA helicase for HIV-1 Rev-RRE export function.* Cell, 2004. **119**(3): p. 381-92.

103. Janda, M. and P. Ahlquist, *RNA-dependent replication, transcription, and persistence of brome mosaic virus RNA replicons in S. cerevisiae.* Cell, 1993. **72**(6): p. 961-70.

104. Beckham, C.J., et al., *Interactions between brome mosaic virus RNAs and cytoplasmic processing bodies.* J Virol, 2007. **81**(18): p. 9759-68.

105. Pillai, R.S., *MicroRNA function: multiple mechanisms for a tiny RNA?* RNA, 2005. **11**(12): p. 1753-61.

106. Mok, B.W., et al., *The NS1 protein of influenza A virus interacts with cellular processing bodies and stress granules through RNA-associated protein 55 (RAP55) during virus infection.* J Virol, 2012. **86**(23): p. 12695-707.

107. Bellocq, C. and D. Kolakofsky, *Translational requirement for La Crosse virus S-mRNA synthesis: a possible mechanism.* J Virol, 1987. **61**(12): p. 3960-7.

108. Schoenberg, D.R. and L.E. Maquat, *Regulation of cytoplasmic mRNA decay.* Nat Rev Genet, 2012. **13**(4): p. 246-59.

109. Baker, K.E. and R. Parker, *Nonsense-mediated mRNA decay: terminating erroneous gene expression.* Curr Opin Cell Biol, 2004. **16**(3): p. 293-9.

110. Chang, Y.F., J.S. Imam, and M.F. Wilkinson, *The nonsense-mediated decay RNA surveillance pathway.* Annu Rev Biochem, 2007. **76**: p. 51-74.

111. Maquat, L.E., W.Y. Tarn, and O. Isken, *The pioneer round of translation: features and functions.* Cell, 2010. **142**(3): p. 368-74.

112. Hwang, J., et al., *UPF1 association with the cap-binding protein, CBP80, promotes nonsense-mediated mRNA decay at two distinct steps.* Mol Cell, 2010. **39**(3): p. 396-409.

113. Wittmann, J., E.M. Hol, and H.M. Jack, *hUPF2 silencing identifies physiologic substrates of mammalian nonsense-mediated mRNA decay.* Mol Cell Biol, 2006. **26**(4): p. 1272-87.

114. Mendell, J.T., et al., *Nonsense surveillance regulates expression of diverse classes of mammalian transcripts and mutes genomic noise.* Nat Genet, 2004. **36**(10): p. 1073-8.

115. Isken, O., et al., *Upf1 phosphorylation triggers translational repression during nonsense-mediated mRNA decay.* Cell, 2008. **133**(2): p. 314-27.

116. Okada-Katsuhata, Y., et al., *N- and C-terminal Upf1 phosphorylations create binding platforms for SMG-6 and SMG-5:SMG-7 during NMD.* Nucleic Acids Res, 2012. **40**(3): p. 1251-66.

117. Buhler, M., et al., *EJC-independent degradation of nonsense immunoglobulin-mu mRNA depends on 3' UTR length.* Nat Struct Mol Biol, 2006. **13**(5): p. 462-4.

118. Eberle, A.B., et al., *SMG6 promotes endonucleolytic cleavage of nonsense mRNA in human cells.* Nat Struct Mol Biol, 2009. **16**(1): p. 49-55.

119. LeBlanc, J.J. and K.L. Beemon, *Unspliced Rous sarcoma virus genomic RNAs are translated and subjected to nonsense-mediated mRNA decay before packaging.* J Virol, 2004. **78**(10): p. 5139-46.

120. Tani, H., M. Torimura, and N. Akimitsu, *The RNA degradation pathway regulates the function of GAS5 a non-coding RNA in mammalian cells.* PLoS One, 2013. **8**(1): p. e55684.

121. Brogna, S., P. Ramanathan, and J. Wen, *UPF1 P-body localization.* Biochem Soc Trans, 2008. **36**(Pt 4): p. 698-700.

122. Bruno, I. and M.F. Wilkinson, *P-bodies react to stress and nonsense.* Cell, 2006. **125**(6): p. 1036-8.

123. Tharun, S. and R. Parker, *Analysis of mutations in the yeast mRNA decapping enzyme.* Genetics, 1999. **151**(4): p. 1273-85.

124. She, M., et al., *Crystal structure of Dcp1p and its functional implications in mRNA decapping.* Nat Struct Mol Biol, 2004. **11**(3): p. 249-56.

125. Li, Y., M. Song, and M. Kiledjian, *Differential utilization of decapping enzymes in mammalian mRNA decay pathways.* RNA, 2011. **17**(3): p. 419-28.

126. Gu, M. and C.D. Lima, *Processing the message: structural insights into capping and decapping mRNA.* Curr Opin Struct Biol, 2005. **15**(1): p. 99-106.

127. He, F. and A. Jacobson, *Identification of a novel component of the nonsense-mediated mRNA decay pathway by use of an interacting protein screen.* Genes Dev, 1995. **9**(4): p. 437-54.

128. Lykke-Andersen, J., *Identification of a human decapping complex associated with hUpf proteins in nonsense-mediated decay.* Mol Cell Biol, 2002. **22**(23): p. 8114-21.

129. Schmaljohn, C.S., *Molecular biology of hantaviruses*. 1996, New York, NY.: Plenum Press.

130. Schmaljohn CS, H.J., *Bunyaviridae: the viruses and their replication*, in *Fields Virology*, H.P. Knipe DM, Editor. 2001, Lippincott, Williams and Wilkins: Philadelphia, PA.

131. Schmaljohn CS, J.C., *Replication of hantaviruses*, in *Hantaviruses*, N.S. Schmaljohn CS, Editor. 2001, Springer-Verlag: Berlin, Germany.

132. Obijeski, J.F., et al., *Structural proteins of La Crosse virus.* J Virol, 1976. **19**(3): p. 985-97.

133. Patterson, J.L. and D. Kolakofsky, *Characterization of La Crosse virus small-genome transcripts.* J Virol, 1984. **49**(3): p. 680-5.

134. Raju, R. and D. Kolakofsky, *The ends of La Crosse virus genome and antigenome RNAs within nucleocapsids are base paired.* J Virol, 1989. **63**(1): p. 122-8.

135. Alfadhli, A., et al., *Hantavirus nucleocapsid protein oligomerization.* J Virol, 2001. **75**(4): p. 2019-23.

136. Alfadhli, A., et al., *Hantavirus nucleocapsid protein coiled-coil domains.* J Biol Chem, 2002. **277**(30): p. 27103-8.

137. Blakqori, G., et al., *Functional L polymerase of La Crosse virus allows in vivo reconstitution of recombinant nucleocapsids.* J Gen Virol, 2003. **84**(Pt 5): p. 1207-14.

138. Bridgen, A. and R.M. Elliott, *Rescue of a segmented negative-strand RNA virus entirely from cloned complementary DNAs.* Proc Natl Acad Sci U S A, 1996. **93**(26): p. 15400-4.

139. Ikegami, T., C.J. Peters, and S. Makino, *Rift valley fever virus nonstructural protein NSs promotes viral RNA replication and transcription in a minigenome system.* J Virol, 2005. **79**(9): p. 5606-15.

140. Kohl, A., et al., *A bunyamwera virus minireplicon system in mosquito cells.* J Virol, 2004. **78**(11): p. 5679-85.

141. Mir, M.A., et al., *Hantavirus N protein exhibits genus-specific recognition of the viral RNA panhandle.* J Virol, 2006. **80**(22): p. 11283-92.

142. Pinschewer, D.D., M. Perez, and J.C. de la Torre, *Role of the virus nucleoprotein in the regulation of lymphocytic choriomeningitis virus transcription and RNA replication.* J Virol, 2003. **77**(6): p. 3882-7.

143. Taylor, S.L., et al., *Hantaan virus nucleocapsid protein binds to importin alpha proteins and inhibits tumor necrosis factor alpha-induced activation of nuclear factor kappa B.* J Virol, 2009. **83**(3): p. 1271-9.

144. Accardi, L., et al., *Activity of Toscana and Rift Valley fever virus transcription complexes on heterologous templates.* J Gen Virol, 2001. **82**(Pt 4): p. 781-5.

145. Dunn, E.F., et al., *Transcription of a recombinant bunyavirus RNA template by transiently expressed bunyavirus proteins.* Virology, 1995. **211**(1): p. 133-43.

146. Lopez, N., et al., *The L protein of Rift Valley fever virus can rescue viral ribonucleoproteins and transcribe synthetic genome-like RNA molecules.* J Virol, 1995. **69**(7): p. 3972-9.

147. Barr, J.N. and G.W. Wertz, *Bunyamwera bunyavirus RNA synthesis requires cooperation of 3'- and 5'-terminal sequences.* J Virol, 2004. **78**(3): p. 1129-38.

148. Crow, M., et al., *Mutational analysis of the influenza virus cRNA promoter and identification of nucleotides critical for replication.* J Virol, 2004. **78**(12): p. 6263-70.

149. Braam, J., I. Ulmanen, and R.M. Krug, *Molecular model of a eucaryotic transcription complex: functions and movements of influenza P proteins during capped RNA-primed transcription.* Cell, 1983. **34**(2): p. 609-18.

150. Caton, A.J. and J.S. Robertson, *Structure of the host-derived sequences present at the 5' ends of influenza virus mRNA.* Nucleic Acids Res, 1980. **8**(12): p. 2591-603.

151. Dhar, R., R.M. Chanock, and C.J. Lai, *Nonviral oligonucleotides at the 5' terminus of cytoplasmic influenza viral mRNA deduced from cloned complete genomic sequences.* Cell, 1980. **21**(2): p. 495-500.

152. Plotch, S.J., et al., *A unique cap(m7GpppXm)-dependent influenza virion endonuclease cleaves capped RNAs to generate the primers that initiate viral RNA transcription.* Cell, 1981. **23**(3): p. 847-58.

153. Yuan, P., et al., *Crystal structure of an avian influenza polymerase PA(N) reveals an endonuclease active site.* Nature, 2009. **458**(7240): p. 909-13.

154. Bishop, D.H., M.E. Gay, and Y. Matsuoko, *Nonviral heterogeneous sequences are present at the 5' ends of one species of snowshoe hare bunyavirus S complementary RNA.* Nucleic Acids Res, 1983. **11**(18): p. 6409-18.

155. Krug, R.M., *Priming of influenza viral RNA transcription by capped heterologous RNAs.* Curr Top Microbiol Immunol, 1981. **93**: p. 125-49.

156. Raju, R., et al., *Nontemplated bases at the 5' ends of Tacaribe virus mRNAs.* Virology, 1990. **174**(1): p. 53-9.

157. Bouloy, M., et al., *Characterization of the 5' and 3' ends of viral messenger RNAs isolated from BHK21 cells infected with Germiston virus (Bunyavirus).* Virology, 1990. **175**(1): p. 50-8.

158. Eshita, Y., et al., *Analyses of the mRNA transcription processes of snowshoe hare bunyavirus S and M RNA species.* J Virol, 1985. **55**(3): p. 681-9.

159. Jin, H. and R.M. Elliott, *Non-viral sequences at the 5' ends of Dugbe nairovirus S mRNAs.* J Gen Virol, 1993. **74 (Pt 10)**: p. 2293-7.

160. Patterson, J.L., B. Holloway, and D. Kolakofsky, *La Crosse virions contain a primer-stimulated RNA polymerase and a methylated cap-dependent endonuclease.* J Virol, 1984. **52**(1): p. 215-22.

161. Simons, J.F. and R.F. Pettersson, *Host-derived 5' ends and overlapping complementary 3' ends of the two mRNAs transcribed from the ambisense S segment of Uukuniemi virus.* J Virol, 1991. **65**(9): p. 4741-8.

162. Hutchinson, K.L., C.J. Peters, and S.T. Nichol, *Sin Nombre virus mRNA synthesis.* Virology, 1996. **224**(1): p. 139-49.

163. Morin, B., et al., *The N-terminal domain of the arenavirus L protein is an RNA endonuclease essential in mRNA transcription.* PLoS Pathog, 2010. **6**(9): p. e1001038.

164. Parker, R. and H. Song, *The enzymes and control of eukaryotic mRNA turnover.* Nat Struct Mol Biol, 2004. **11**(2): p. 121-7.

165. Eulalio, A., I. Behm-Ansmant, and E. Izaurralde, *P bodies: at the crossroads of post-transcriptional pathways.* Nat Rev Mol Cell Biol, 2007. **8**(1): p. 9-22.

166. Meyer, S., C. Temme, and E. Wahle, *Messenger RNA turnover in eukaryotes: pathways and enzymes.* Crit Rev Biochem Mol Biol, 2004. **39**(4): p. 197-216.

167. Isken, O. and L.E. Maquat, *Quality control of eukaryotic mRNA: safeguarding cells from abnormal mRNA function.* Genes Dev, 2007. **21**(15): p. 1833-56.

168. Amrani, N., M.S. Sachs, and A. Jacobson, *Early nonsense: mRNA decay solves a translational problem.* Nat Rev Mol Cell Biol, 2006. **7**(6): p. 415-25.

169. Conti, E. and E. Izaurralde, *Nonsense-mediated mRNA decay: molecular insights and mechanistic variations across species.* Curr Opin Cell Biol, 2005. **17**(3): p. 316-25.

170. Lejeune, F. and L.E. Maquat, *Mechanistic links between nonsense-mediated mRNA decay and pre-mRNA splicing in mammalian cells.* Curr Opin Cell Biol, 2005. **17**(3): p. 309-15.

171. Cao, D. and R. Parker, *Computational modeling and experimental analysis of nonsense-mediated decay in yeast.* Cell, 2003. **113**(4): p. 533-45.

172. Mitchell, P. and D. Tollervey, *An NMD pathway in yeast involving accelerated deadenylation and exosome-mediated 3'-->5' degradation.* Mol Cell, 2003. **11**(5): p. 1405-13.

173. Rao, P., W. Yuan, and R.M. Krug, *Crucial role of CA cleavage sites in the cap-snatching mechanism for initiating viral mRNA synthesis.* EMBO J, 2003. **22**(5): p. 1188-98.

174. Banerjee, A.K., *5'-terminal cap structure in eucaryotic messenger ribonucleic acids.* Microbiol Rev, 1980. **44**(2): p. 175-205.

175. Yang, Z., et al., *GW182 is critical for the stability of GW bodies expressed during the cell cycle and cell proliferation.* J Cell Sci, 2004. **117**(Pt 23): p. 5567-78.

176. Raju, R. and D. Kolakofsky, *Inhibitors of protein synthesis inhibit both La Crosse virus S-mRNA and S genome syntheses in vivo.* Virus Res, 1986. **5**(1): p. 1-9.

177. Raju, R. and D. Kolakofsky, *Unusual transcripts in La Crosse virus-infected cells and the site for nucleocapsid assembly.* J Virol, 1987. **61**(3): p. 667-72.

178. Duijsings, D., R. Kormelink, and R. Goldbach, *In vivo analysis of the TSWV cap-snatching mechanism: single base complementarity and primer length requirements.* EMBO J, 2001. **20**(10): p. 2545-52.

179. Krautkramer, E., M. Zeier, and A. Plyusnin, *Hantavirus infection: an emerging infectious disease causing acute renal failure.* Kidney Int, 2013. **83**(1): p. 23-7.

180. Plyusnina, A., et al., *Genetic characterization of seoul hantavirus originated from norway rats (Rattus norvegicus) captured in Belgium.* J Med Virol, 2012. **84**(8): p. 1298-303.

181. Razzauti, M., et al., *Microevolution of Puumala hantavirus during a complete population cycle of its host, the bank vole (Myodes glareolus).* PLoS One, 2013. **8**(5): p. e64447.

182. Vaheri, A., et al., *Hantavirus infections in Europe and their impact on public health.* Rev Med Virol, 2013. **23**(1): p. 35-49.

183. Hepojoki, J., et al., *Hantavirus structure--molecular interactions behind the scene.* J Gen Virol, 2012. **93**(Pt 8): p. 1631-44.

184. Battisti, A.J., et al., *Structural studies of Hantaan virus.* J Virol, 2011. **85**(2): p. 835-41.

185. Fuerst, T.R., et al., *Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase.* Proc Natl Acad Sci U S A, 1986. **83**(21): p. 8122-6.

186. Matsuo, E. and P. Roy, *Bluetongue virus VP6 acts early in the replication cycle and can form the basis of chimeric virus formation.* J Virol, 2009. **83**(17): p. 8842-8.

187. de Wit, E., et al., *A reverse-genetics system for Influenza A virus using T7 RNA polymerase.* J Gen Virol, 2007. **88**(Pt 4): p. 1281-7.

188. Freiberg, A., et al., *Establishment and characterization of plasmid-driven minigenome rescue systems for Nipah virus: RNA polymerase I- and T7-catalyzed generation of functional paramyxoviral RNA.* Virology, 2008. **370**(1): p. 33-44.

189. Ulmanen, I., B.A. Broni, and R.M. Krug, *Role of two of the influenza virus core P proteins in recognizing cap 1 structures (m7GpppNm) on RNAs and in initiating viral RNA transcription.* Proc Natl Acad Sci U S A, 1981. **78**(12): p. 7355-9.

190. Cheng, E. and M.A. Mir, *Signatures of host mRNA 5' terminus for efficient hantavirus cap snatching.* J Virol, 2012. **86**(18): p. 10173-85.

191. Brown, K.S., H. Ebihara, and H. Feldmann, *Development of a minigenome system for Andes virus, a New World hantavirus.* Arch Virol, 2012. **157**(11): p. 2227-33.

192. Doan, L., et al., *Metal ion catalysis of RNA cleavage by the influenza virus endonuclease.* Biochemistry, 1999. **38**(17): p. 5612-9.

193. Crepin, T., et al., *Mutational and metal binding analysis of the endonuclease domain of the influenza virus polymerase PA subunit.* J Virol, 2010. **84**(18): p. 9096-104.

194. Hopkins, K.C., et al., *A genome-wide RNAi screen reveals that mRNA decapping restricts bunyaviral replication by limiting the pools of Dcp2-accessible targets for cap-snatching.* Genes Dev, 2013. **27**(13): p. 1511-25.

195. Vaheri, A., et al., *Uncovering the mysteries of hantavirus infections.* Nature Rev Microbio, 2013. **11**(8): p. 539-50.